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METABOLISM, MASS SPECTRAL ANALYSIS AND MODE OF ACTION OF TRICHOTHECENE MYCOTOXINS

ANNUAL REPORT

15 JULY 1986 THROUGH 14 JULY 1987
15 SEPTEMBER 1987

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An analytical method for T-2, HT-2 and T-2-tetraol using multiple reaction monitoring (MRM) was initiated. The appropriate parent and daughter ions were selected. The sensitivity of the method is one part per billion. An isomer of HT-2 was detected. The mass spectra of H-1 (parent and daughter ions) were obtained. Toxic Fusarium isolates were identified.

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SUMMARY

The development of an analytical method for the analysis of T-2, HT-2 and T-2-tetraol in blood and urine was initiated. The objective is to use multiple reaction monitoring (MRM) facility of the VG 7070EQ tandem mass spectrometer. The advantage of the latter is that the noise from the biological matrix is kept at a minimum and as a result the sensitivity is increased. Essentially, the magnet is parked on the parent ion and only this fragment is allowed to pass through the first mass spectrometer. After the selected fragment passes into the quadrupole #1, it is decomposed into fragments by collision activated (in argon plasma and 20 30 volts) decomposition. The daughter ions are detected by quadrupole #2. Using this method, T-2 and HT-2 can be detected at one part per billion. Keywords: T-2 Toxin, HT-2 Toxin

In the development of the MRM method of analysis for T-2-TFA, we discovered an interfering parent fragment of 478 that had a retention time close to that of T-2. The substance did not present a problem because its daughters were different from that of T-2-TFA. This also illustrates that although the parents of a substance are the same, the daughters are not.

The fragmentation map of the TFA derivatives of T-2, HT-2 and T-2-tetraol as well as their daughter ion sepctra have been obtained.

An isomer of HT-2 (C-4 acetate) was discovered in a Fusarium culture using the daughter ions of HT-2 as a probe. Its structure was determined using parent-daughter mass spectra. The detection of the isomer in the Fusarium culture, composed of a vast mixture of compounds, illustrates the utility of the daughter ion library as a practical analytical probe.

The daughter ion fragments for T-2, HT-2 and T-2-tetraol were determined using respectively parents m/z+ 478, 532, and 568. T-2-tetraol can also be detected by using the parent 330 which yields the daughter 216.

This report also lists all the Fusarium species isolated from Norway and New Zealand and the toxins they produce. Those from Norway almost exclusively produce the hemorrhagic toxin called H-1. This toxin was also found in New Zealand on the South Island.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

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MULTIPLE REACTION MONITORING (MRM) WITH T-2 TOXIN, HT-2 TOXIN AND T-2-TETRAOL

In Quarterly Report number VI, we reported on the analysis of T-2 toxin in a blood matrix using the MRM procedure with the VG7070EQ instrument. We were able to obtain what should be the maximum in selectivity and sensitivity of the scanning portions of the instrument. MRM is a tandum mass spectral scanning technique that forces the electro static analyzer and analyzers of the magnet and quadrupole to focus on specific reactions (parents to daughter ions) and thereby increase sensitivity and selectivity. We report here the expansion of this technique to to include HT-2 toxin and T-2-tetraol.

Selectivity of MRM relies on specific parent and daughter ion relationships. Parent ions of a certain molecule will produce unique unambiguous daughter ions of precise abundance using a specified set of reaction conditions. The daughter and parent ions taken together make up what is known as a fragmentation map. The latter is a pathway of decomposition of the ionized molecule and suggests the structure of the molecule . They are also valuable analytically. The majority of the total ion current will reside within only a few of the fragments usually found in the low mass region. Normally the low mass region would have many interfering substances from the biological matrix but in the case of MRM, this fault is eliminated. The reason for this is that only a single parent is allowed through the analyzer and on to the collision chamber where the daughter ions are formed. Thus the MRM procedure is well suited to take full advantage of high abundance ions as well as highly specific reactions.

ANALYSIS OF HT-2 TOXIN: The fragmentation map and daughter ion spectra of HT-2-(TFA) is presented in figures 1-5. Most of the abundance of the ion current is located in fragments 121, 138 and 180. These ions are daughters of parent 532. They are presumed to have the same structure as the same daughters found in T-2-TFA but derived from parent ion 478. Both parent ions (532 for HT-2 and 478 for T-2) are formed from a loss of 84 mass units from the molecular ion. Since these are high abundance ions and are formed from a significant precursor of the molecules intact skeleton, they are an excellent choice for MRM analysis. Figure 6 shows the results of analysis of HT-2 from urine using daughters 180, 138 and 121. Note the prominant ions and lack of noise from the biological matrix.

DETECTION OF AN ISOMER OF HT-2 TOXIN(TFA) USING THE DAUGHTERS 180, 138 AND 121: The fragments of an isomer of HT-2 TFA is shown in figure 14 and 15. The isomerism resides around C-4 carbon and

was detected as shown in the caption of figure 14. The diagnostic ion for the C-4 isomer of HT-2 toxin is m/z+227. Figure 16 shows the positive chemical ionization mass spectrum of iso-HT-2 . The base peak of the most abundant HT-2 isomer (C-15 acetate) is 455. However, the base peak of the iso-HT-2 is 401 just as found in T-2 toxin (figure 19). The fragment 401 is formed by loss of the isovaleroxy group (101) to yield 515 followed by a loss of the C-15 TFA group (114) to yield 401.

ANALYSIS OF T-2-TETRAOL: The daughter ion fragmentation map of T-2-tetraol (figure 7) is different from that described for T-2 and HT-2 toxins. They differ in ion abundance as well as the structure of the primary and secondary fragments. The two most significant reactions are the parent ion 569 going to daughter 455 and parent 330 going to 216. The latter are abundant , reproducable , give the minimum of background signal and are easy to monitor. The MRM analysis of T-2-tetraol extracted from urine is shown in figure 13. The parent ion 330 yields a very prominant daughter in 216. On the other hand, parent 568 yields a very poor daughter at 454. The parent of choice is m/z+569.

CONDITIONS USED IN MRM ANALYSIS: The MRM analyses were done using two reaction groups. The first one covered the retention time of T-2-tetraol and the second covered T-2 and HT-2. The dwell time was 100 milliseconds, with an analyzer switch time of 20 milliseconds. The mass spectrometer tuning and calibration procedures were as described by the manufacturer i.e. calibrate the front end first followed by the quad. The electron voltage was 70, collision cell voltage was14-16 volts, the collision cell argon pressure was 10-6 torr.

The derivatives used were trifluoroacetate made from trifluoro-acetic acid anhydride. The capillary column was a DB-5 (J&W Scientific) 0.25 mm id. The column temperature and program was 80 to 300 degreees C. at 25 degrees per minute.

The urine was spiked with an equivalent to give 50 parts per billion. Samples were extracted according to procedures reported in earlier reports using BONDELUTE columns.

STRUCTURE ELUCIDATION OF THE C-4 ACETATE ISOMER OF HT-2 TOXIN:

The C-4 isomer is called iso-HT-2 analagous to isoT-2 toxin.It is 3,15-dihydroxy-4-acetoxy-8-[3-methyl-butyloxy]-12,13-epoxy-tri-chothecec-9-one. The structure was determined by deduction using the mass spectrum and daughter ions formed. The isomer was found in a culture of a Fusarium isolate grown on rice in the laboratory. The primary mass spectra of the TFA derivative in both electron impact and chemical ionization was confirmed as

being 616 (figure 16). The mass spectra were similar but not identical to the C-15 isomer of HT-2 and the C-4 isomer of T-2 toxin. The daughter ion spectra are helpful in identifying the isomer because of the sequence of loss of the isovaleroxy group at C-8 (loss of 101) followed by the loss of the C-15 acetate as in the C-15 isomer (loss of 60) or loss of 114 amu in the case of the C-4 isomer. Thus the C-4 isomer should yield a either m/e+ 515 or 532 followed by either 401 or 413 and finnaly resulting in m/z+ 227. Iso-T-2 toxin, in contradistinction, will yield an m/z+ 287.

The C-15 acetate isomer of HT-2 toxin should yield a m/z+455 because of the loss of an acetate (60amu) from m/z+ 515. The latter is formed after loss of 101 amu from the isovaleroxy group on C-8 (figure 19). The C-4 acetate isomer will lose 114 amu units from the TFA formed at C-15 and yield m/z+401 as in T-2 toxin.

FIGURE 1. Mass spectrum of T-2 TFA derivative. The M+ is 616 and the origin of daughters is as shown in the fragmentation scheme. Thus m/z+ 616 gives rise directly to 532 which in turn is the parent of m/z+ fragments 180, 138 and 121. These are the same daughters as those found in the parent fragment m/z+ 478 of T-2-TFA. The latter daughter fragments are very intense and are the choice for both quantitative and qualitative determination of HT-2-TFA. This is an example where two closely related derivatives have the same daughters but different parents i.e. 532 in HT-2 and 478 in T-2.

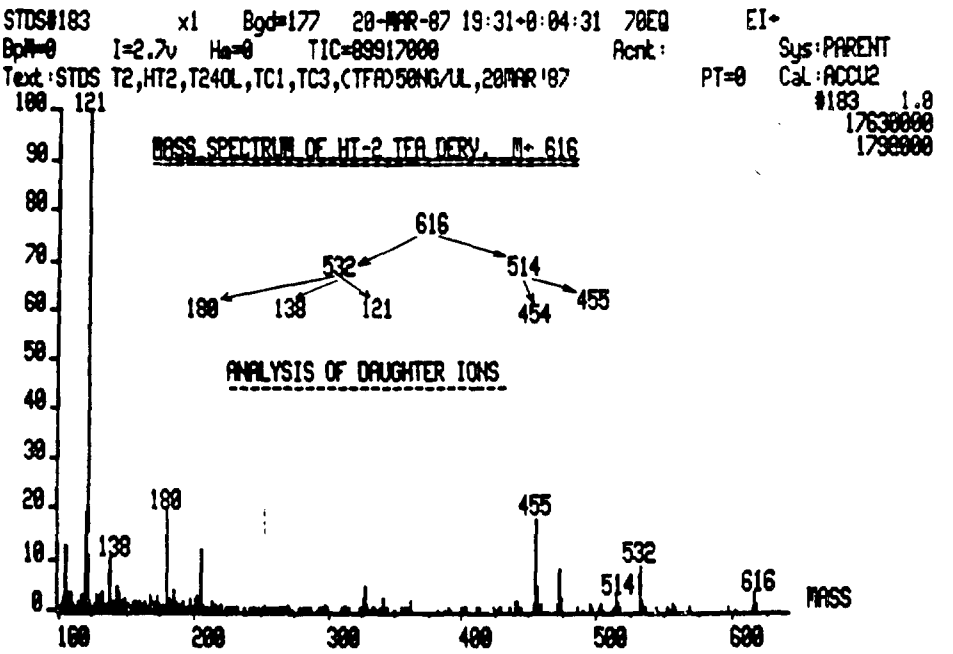


FIGURE 2. Daughter ion mass spectrum of HT-2-TFA using the M+ 616 as the parent fragment. Note that m/z+ 180, 138 and 121 are present in the spectrum but vary in intensity from that of the full scan E.I. mass spectrum shown in figure 1. Daughters 556, 423, 333, 264 and 220 are not found in the full scan mass spectrum indicating that different reactions are occurring under E.I. conditions. Note the m/z+ 455, a classic indicator of HT-2 and its derivatives is not found as a daughter of 616.

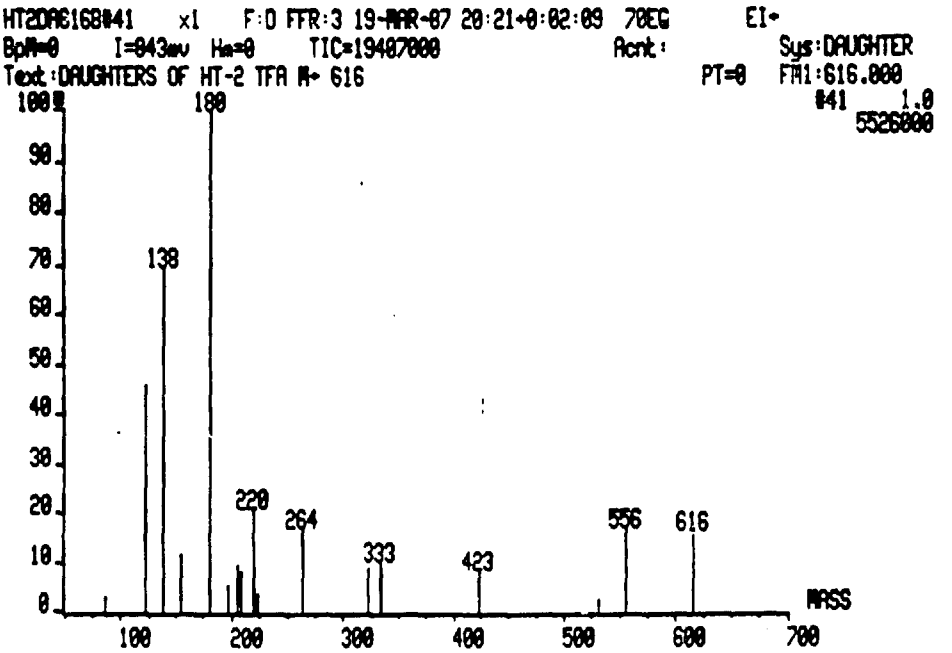


FIGURE 3. Daughter ion mass spectrum of parent m/z+ 532 of HT-2-TFA using argon as the collision plasma. Daughters 121, 138 and 180 are the products of collision activated decomposition of 532. The latter are very intense and stable , hence useful in MRM experiments.

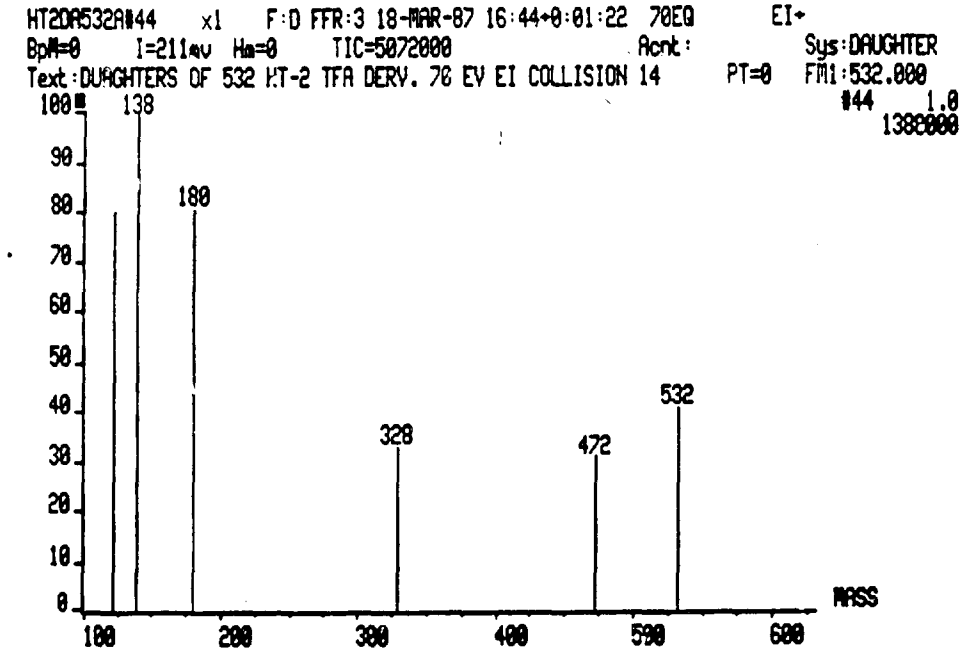


FIGURE 4. Daughter ion mass spectrum of HT-2-TFA using m/z+ 514 as the parent. Note that fragment 454 occurs as the daughter which is analagous to m/z+ 455 of the full scan E.I. spectrum. It is presumed that 455 can be formed just as well as 454 but it depends on which reaction species the hydrogen follows .

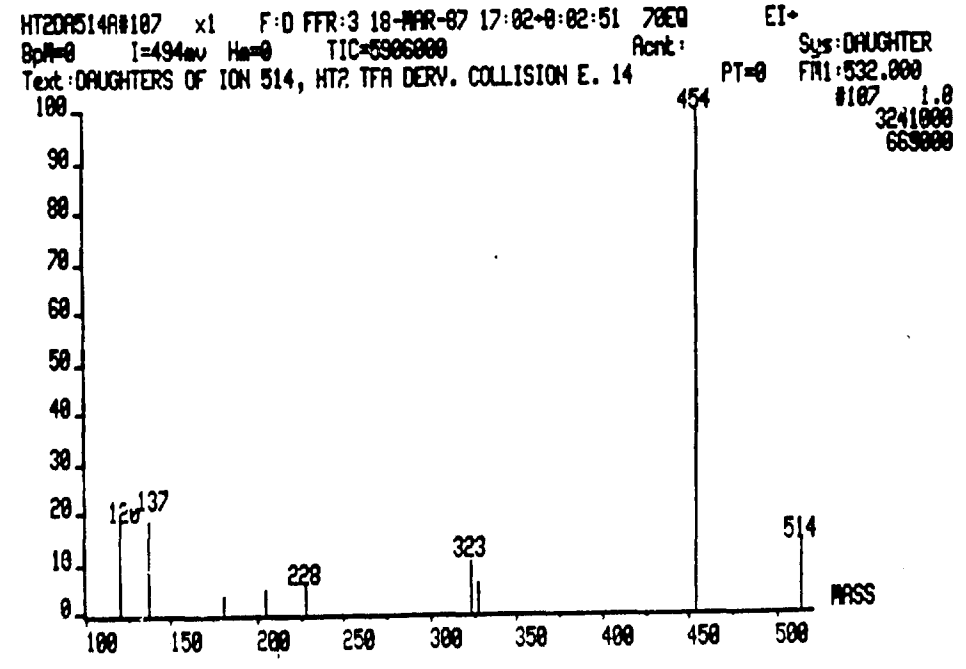


FIGURE 5. Daughter ion mass spectrum of parent 455 of HT-2-TFA. Note that m/z+455 is a stable species with little activity in terms of daughter fragments. This suggests that this fragment has an unsaturated ring structure which normally confers stability i.e. double bonds in the ring are stable.

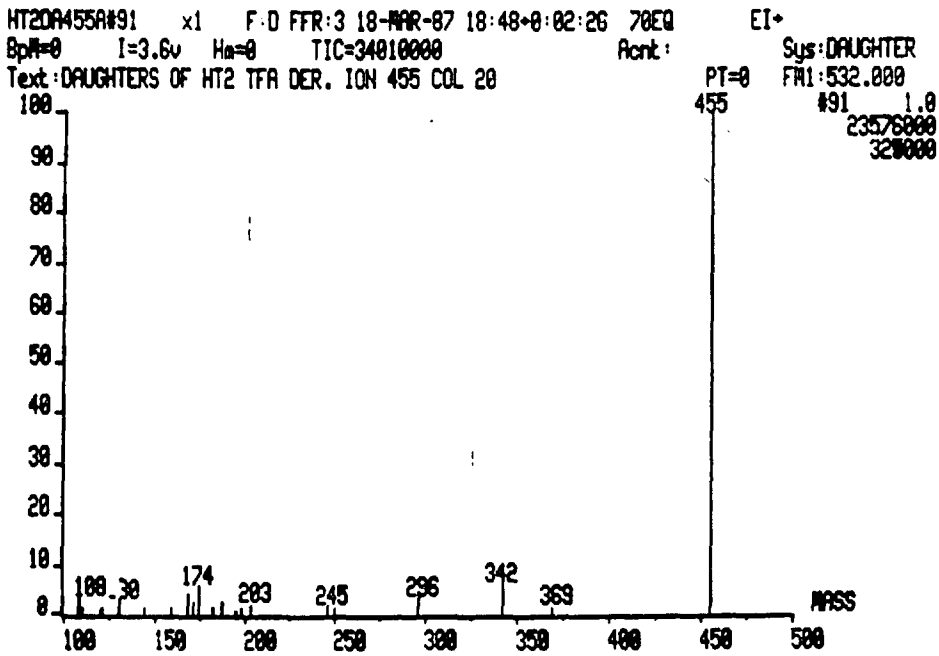
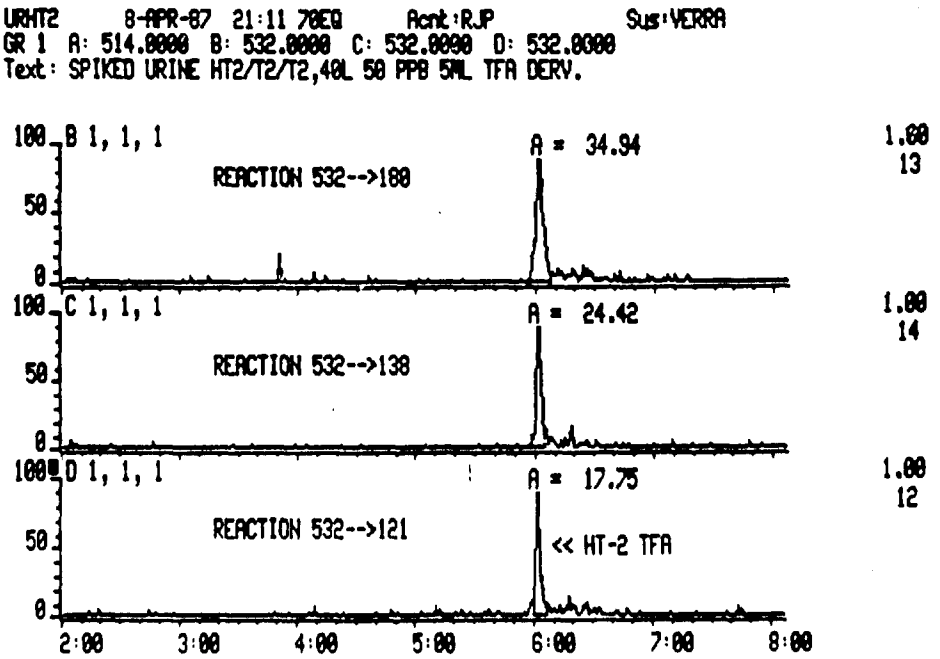


FIGURE 6. Analysis of HT-2-TFA toxin extracted from urine, spiked at 50 ppb, using multiple reaction monitoring. Parent fragment 532 was allowed to pass through the magnet portion of the MS after which it was bombarded with electrons in the presence of argon in the first quad region and scanned for daughters in the second quad region. Boxes B thru D represent the total ion current of m/z+ fragments 180, 138, and 121 respectively.



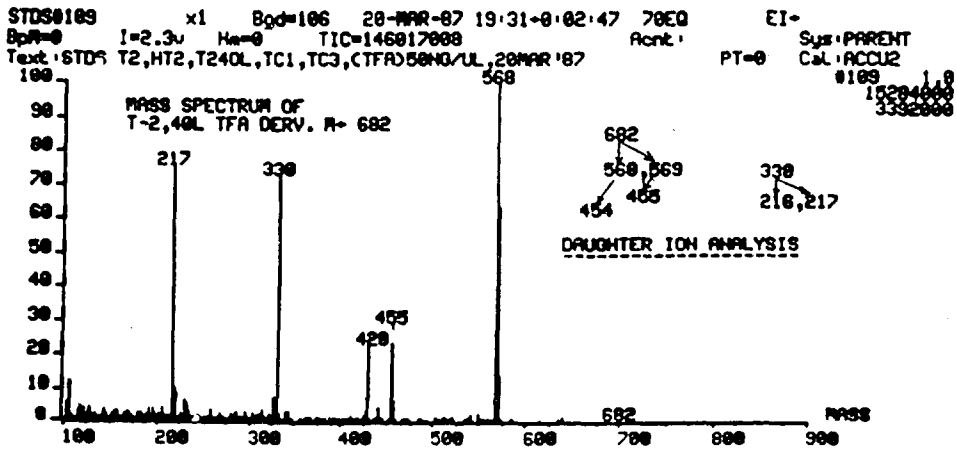


FIGURE 7. Electron impact mass spectrum of the TFA derivative of T-2-tetraol. The scheme of daughter ions and their parents is presented in the right hand corner. The major diagnostic fragments are m/z+ 568, 455, 330 and 217.

FIGURE 8. Daughter ion mass spectrum of fragment 682 (M+) of the TFA derivative of T-2-tetraol. The most important daughter fragment ion is m/z+ 568 which is the base peak of the E.I. spectrum

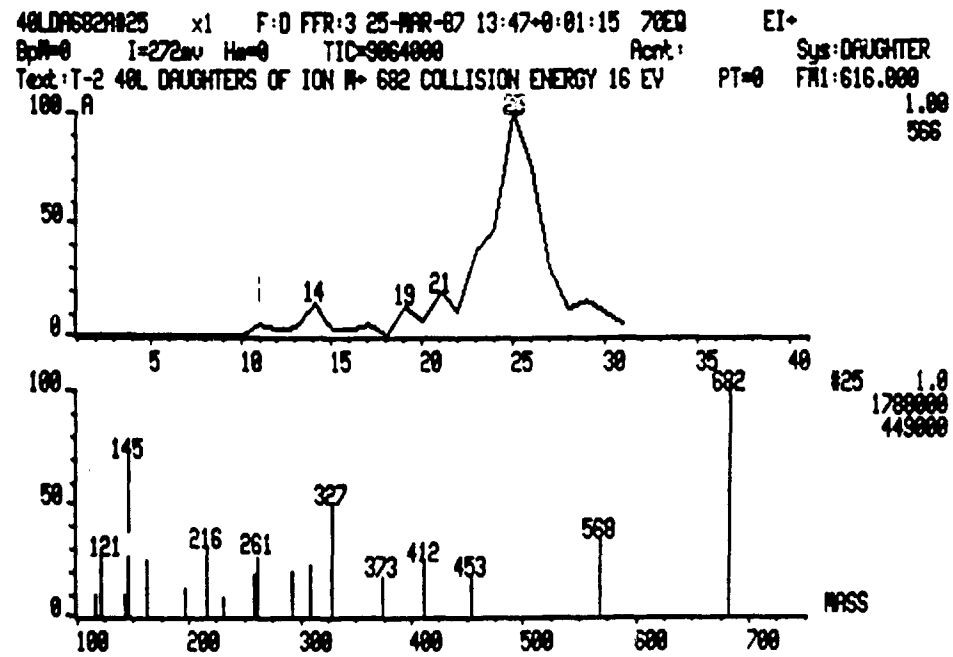


FIGURE 9. Daughter ion mass spectrum of m/z+569 giving rise to m/z+455 and 328.

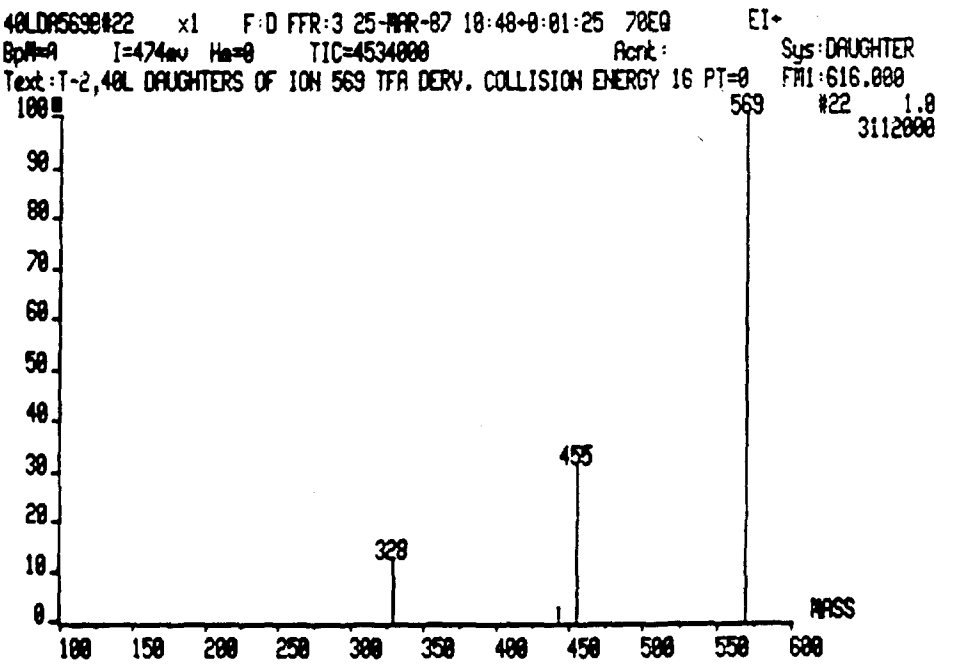


FIGURE 10. Daughter ion mass spectrum of parent fragment 568 and the resulting daughter fragments. Note that m/z+455 (daughter of 569) is not present in this spectrum.

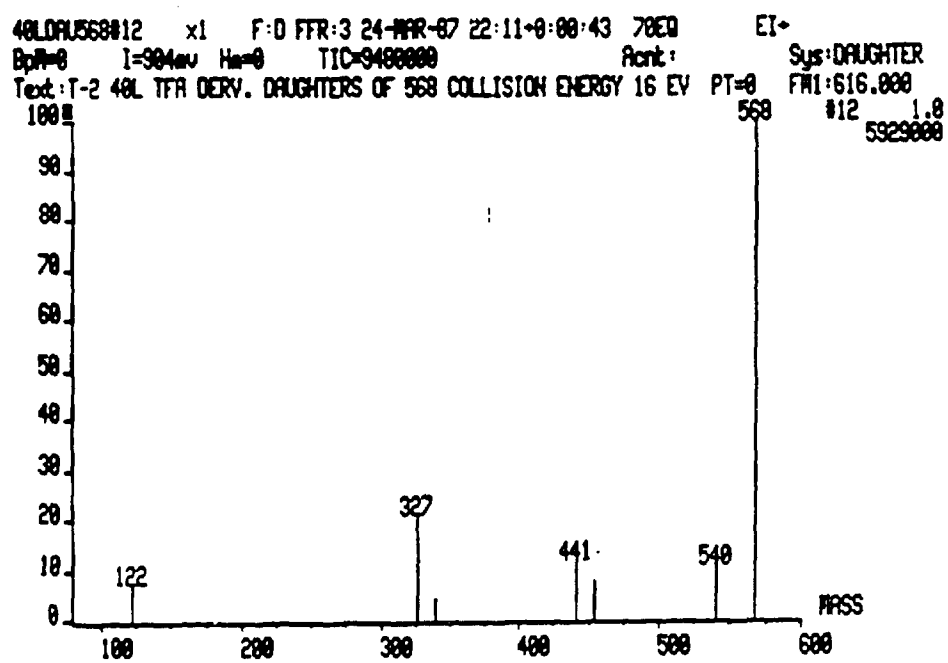


FIGURE 11. Daughter ion mass spectrum of the TFA derivative of T-2-tetraol using m/z+ 455 as the parent. Although there are a number of low intensity daughters, none are of interest in the use of MRM.

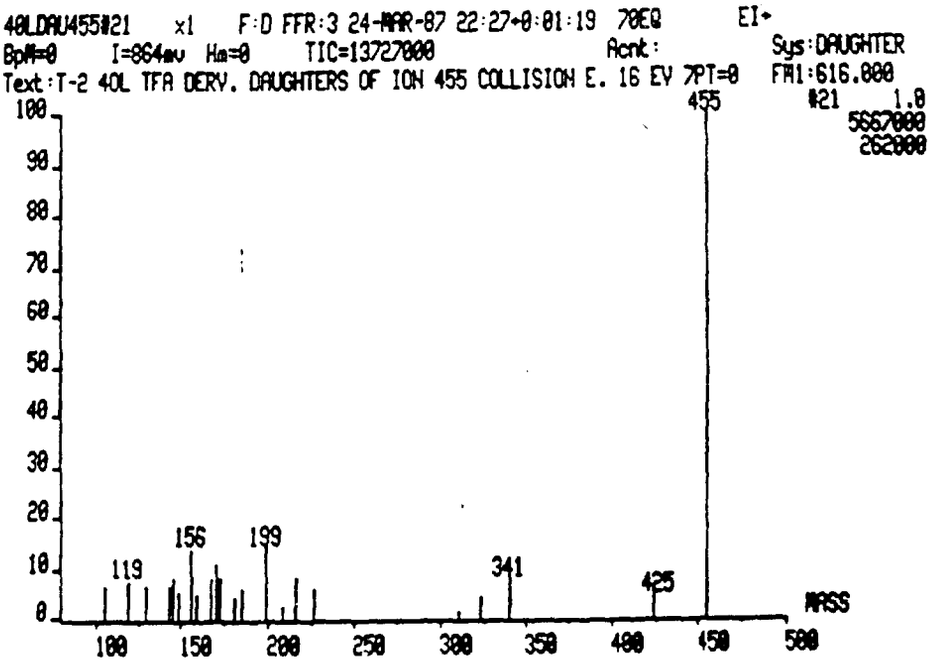


FIGURE 12. Daughter ion mass spectrum of the TFA derivative of T-2-tetraol parent fragment 330. The resulting daughter is 216 which is an important and useful fragment for MRM. We recommend its use for MRM of T-2-tetraol.

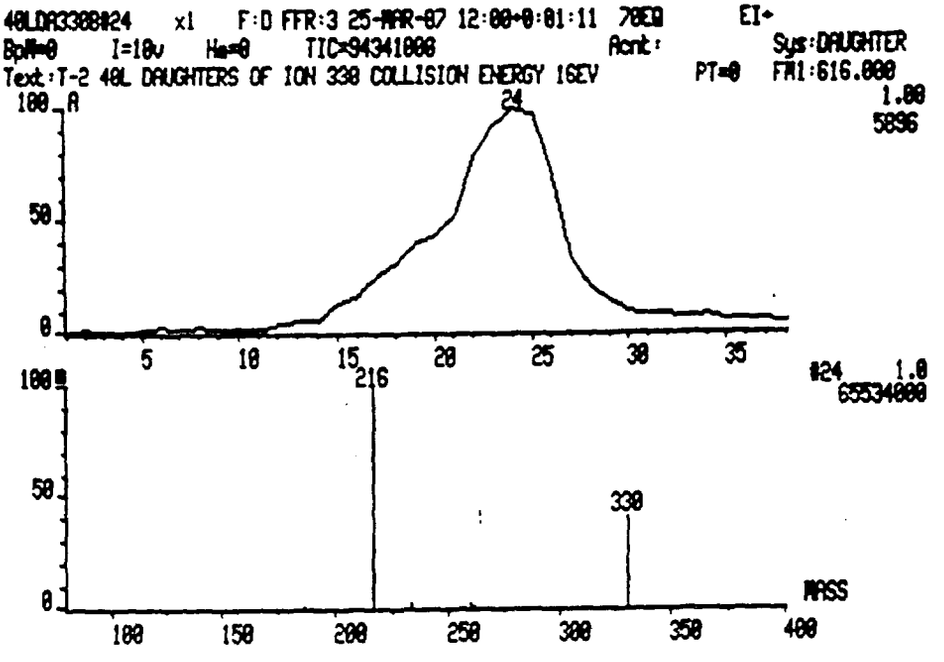


FIGURE 13. Analysis of human urine spiked with T-2-tetraol (TFA) at 50ppb and analyzed by MRM using both 454 and 330 as parent ions. Box A shows the total ion current obtained when m/z+ 568 is used as the parent. Note the noisy base line which precludes the use of this fragment. On the other hand, box B shows the results of 330 as the parent and 216 as the daughter. Note the excellent base line with a signal to noise ratio of about 100/1 which suggests that the use of of 568 as a parent in a biological matrix will give us sensitivity down to 2ppb.

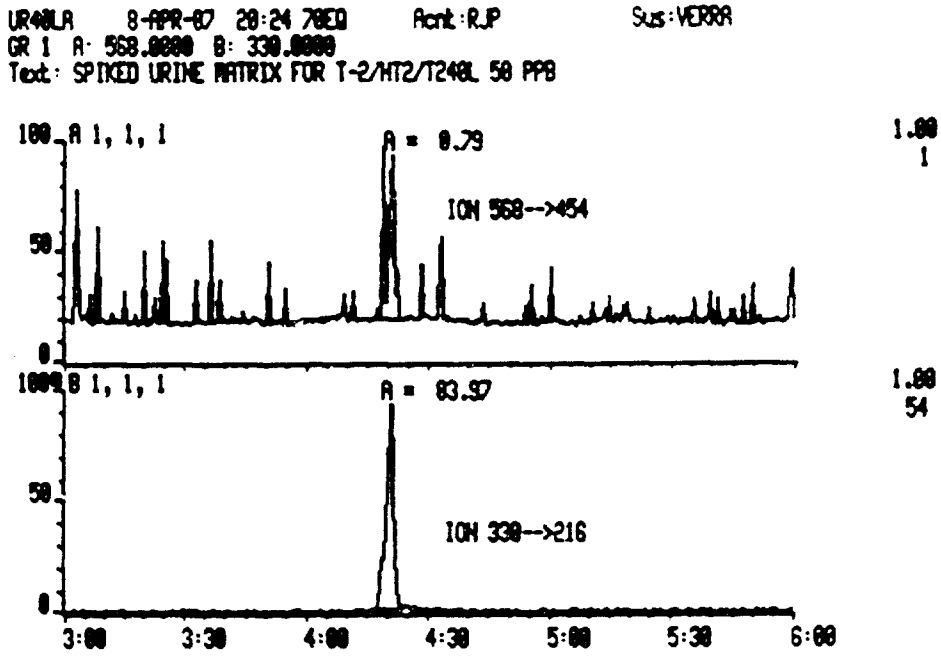


FIGURE 14. Fragments of the TFA derivative of the C-4 isomer (acetate at C-4 instead of C-15) of HT-2 toxin. All of the above fragments and structures have been verified by daughter ion mass spectrometry. TFA-HT-2 toxin will yield a m/z+227 derived from 616 minus the isovaleroxy group to yield m/z+ 515 followed by loss of the TFA from C-15 yielding 401 and then a loss of 60 amu from the C-4 acetate to yield 341 and finally 114 amu from the loss of the C-3 TFA to give 227. The C-15 isomer will also yield m/z+ 227 but from another parent ion. Finally, the C-3 isomer will yield m/z+ 287 by loss of the C-8 group followed by loss of 114 amu each from C-15 and C-4 respectively. Fragmenst 227 and 287 are useful in detecting isomers of T-2, HT-2, TC-1 and TC-3.

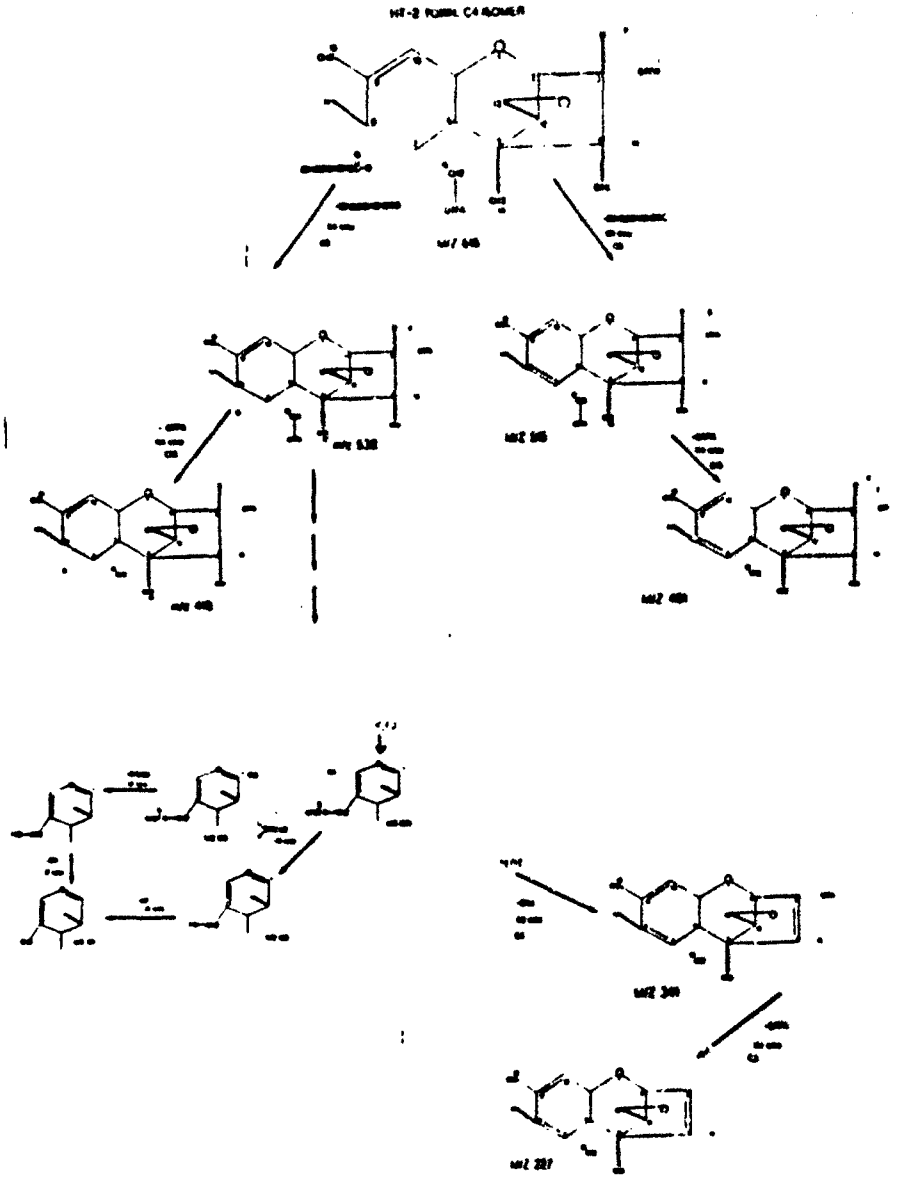


FIGURE 15. Continuation of the breakdown scheme of HT-2 as shown in figure 14. Note that fragments 121, 138 and 180 are unsaturated rings and hence stable in the mass spectrometer. They are further degraded to m/z+ 166 and 121.

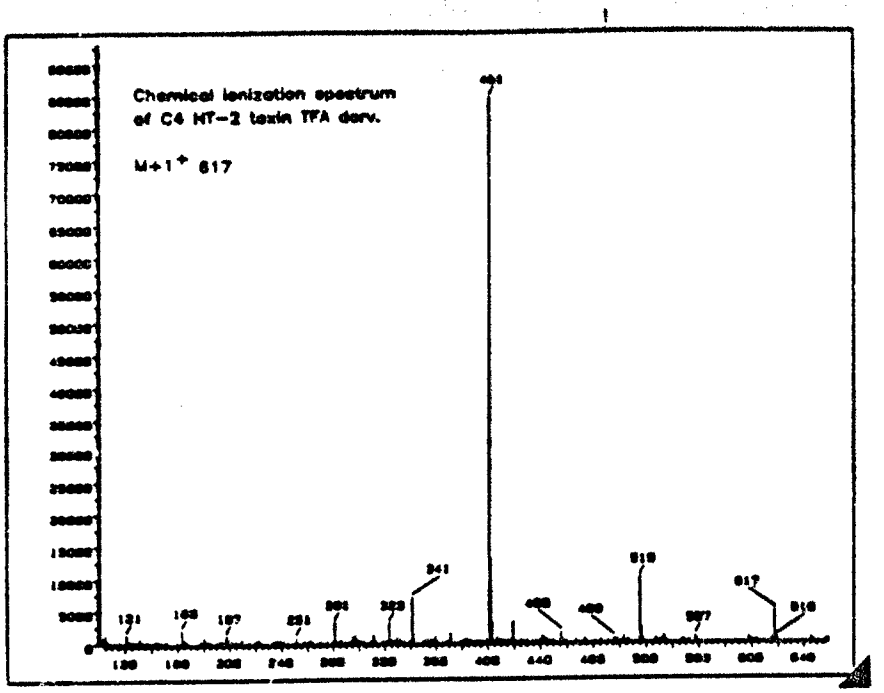


FIGURE 16. Positive chemical ionization mass spectrum of iso-HT-2 toxin (acetate in the C-4 position). Note that the base peak is m/z+ 401 instead of the traditional m/z+455. This is due to the fragmentation of the M+ at 616 which loses the C-8 isovaleroxy to create 515 and then the C-15 TFA (-114) to form m/z+401. The latter loss is characterisitic of TFA-T-2 toxin in PCI.

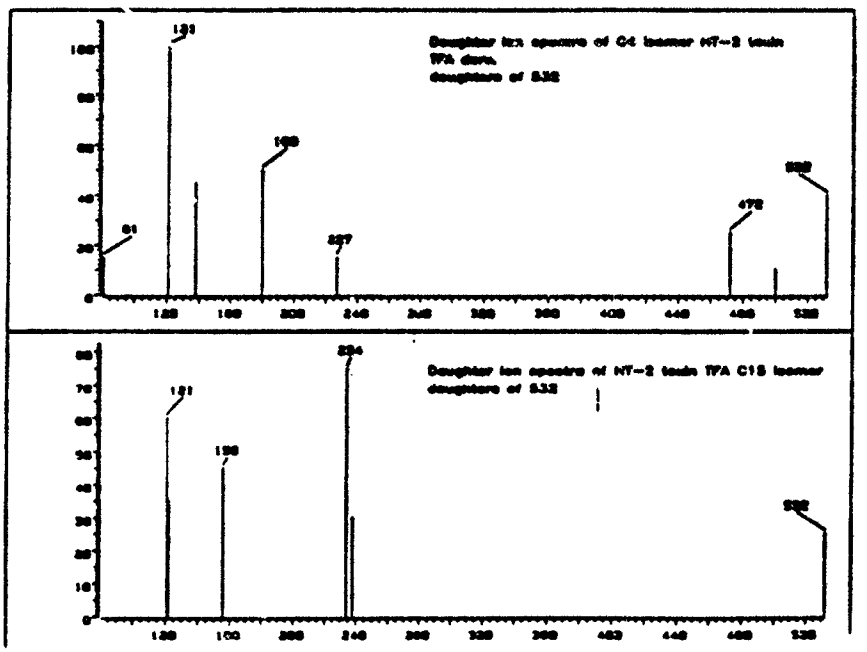


FIGURE 17. Daughter ion mass spectrum of the TFA derivative of HT-2 toxin (C-4 isomer) using m/z+532 as the parent. This is compared with the daughters of m/z+532 from TFA-T-2 toxin. Note the difference in daughters due mainly to the substitution of the TFA group on carbons 15, 4 and 3. The C-15 isomer loses 84 amu to form 532 and then 298 amu (the A ring and the epoxide plus C-3 and C-4) to form the unsaturated B ring at m/z+ 234. See figure 15

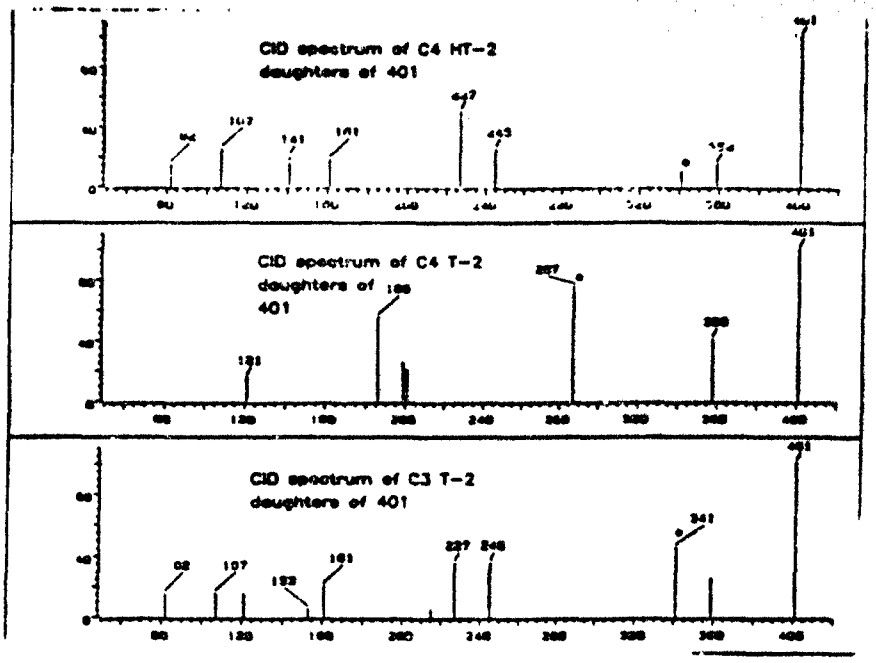


FIGURE 18. Daughter ion mass spectra (TFA derivatives) of the C-4 isomer of HT-2, C-4 isomer of T-2 and the C-3-T-2 toxin. All daughters have been generated from the same parent (m/z+ 401). Although this is true, their structures are not the same. As an example, iso-T-2-TFA should yield m/z+287 (middle box)and does whereas TFA-T-2 should yield m/z+ 327 (bottom box) and does. The daughter ion method is excellent for differentiating this group.

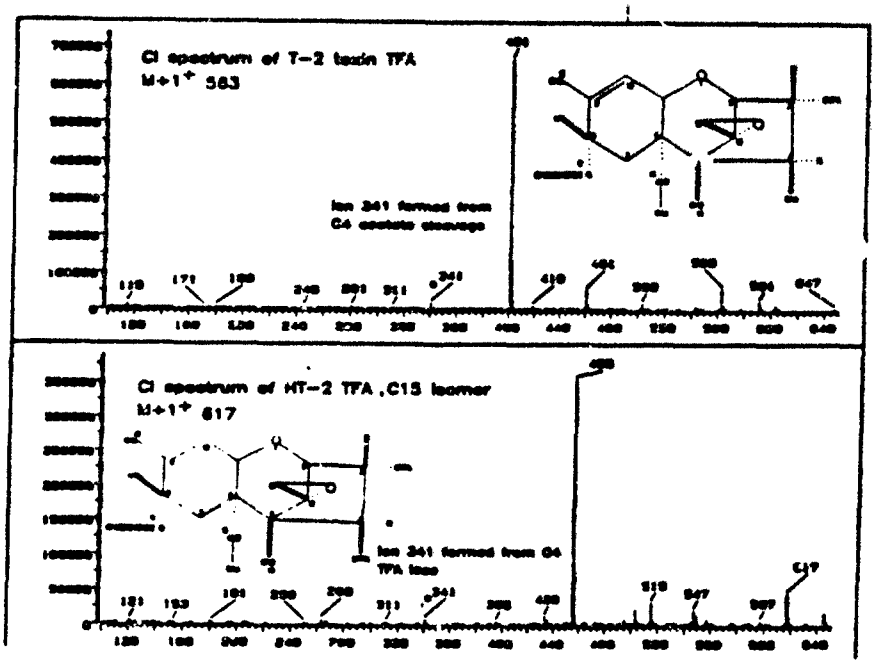


FIGURE 19. Positive chemical ionization (methane) mass spectrum of TFA-T-2 toxin and TFA-HT-2(C-15 isomer). This figure shows that ion 341 of T-2 is formed from 563 by loss of an acetate (60amu) whereas in HT-2 , ion 341 is formed by loss of a TFA group (114) from 616.

IMPLEMENTATION OF T-2 TOXIN ANALYSIS USING MULTIPLE REACTION MONITORING.

The objective of this study is to develop a fool-proof method of analysis of human blood and urine samples for traces of T-2 toxin , HT-2 toxin and T-2-tetraol using multiple mass spectrometry. The reason for this study is to have on record a method to deal with chemical warfare agents such as the trichothecenes so that an unequivocal answer can be given as to their presence in biological samples.

Our criteria are to achieve: 1. ultimate sensitivity (1ppb) and 2. uncompromised detection by the elimination of biological matrix effects. We hope to the surpass the results obtained by use of the Townsend Oxygen Discharge Electrode.

To date we have had good results down to 5ppb using a single daughter ion as the parent for detection of the characteristic fragments. We are studying the daughters in order to determine which of the many daughters lend themselves for good quantitation. A more complete report will be forthcoming in the next quarterly report.

STRUCTURE IDENTITY OF H-1 TOXIN

The mass spectra in both E.I. and C.I. are shown in figures 1 and 2. The molecular ion in EI is 428 and this is confirmed by the molecular ion (428 plus 1) obtained in positive CI (methane). This was also the molecular ion obtained in analysis by Fast Atom Bombardment. Both the EI and CI spectra are rich in large fragment ions in the high mass region of the spectrum. Thus, m/z+ 428, 385, 368, 354, 323 and 296 are very intense and informative ions useful in both interpretation of structure and in quantitation.

The ultraviolet absorbtion spectrum shows 210 and 250 millimicrons as absorption maxima. The UV spectrum does not appear to be too useful.

The infrared spectrum has intense peaks which will prove to be useful in interpretation of the structure after more detailed information is available from NMR analyses. Notable are the absorbtion maxima at 1750, 1650, 1200 reciprocal cm.

Combustion analysis revealed a ratio of carbon, hydrogen and oxygen of 64, 6 and 25% respectively.

Analysis by high resolution mass spectrometry suggest an emperical formula of C23 H24 08.

Structure analysis is being done by NMR. Large sample size preparations are being made in order to facilitate structure identification.

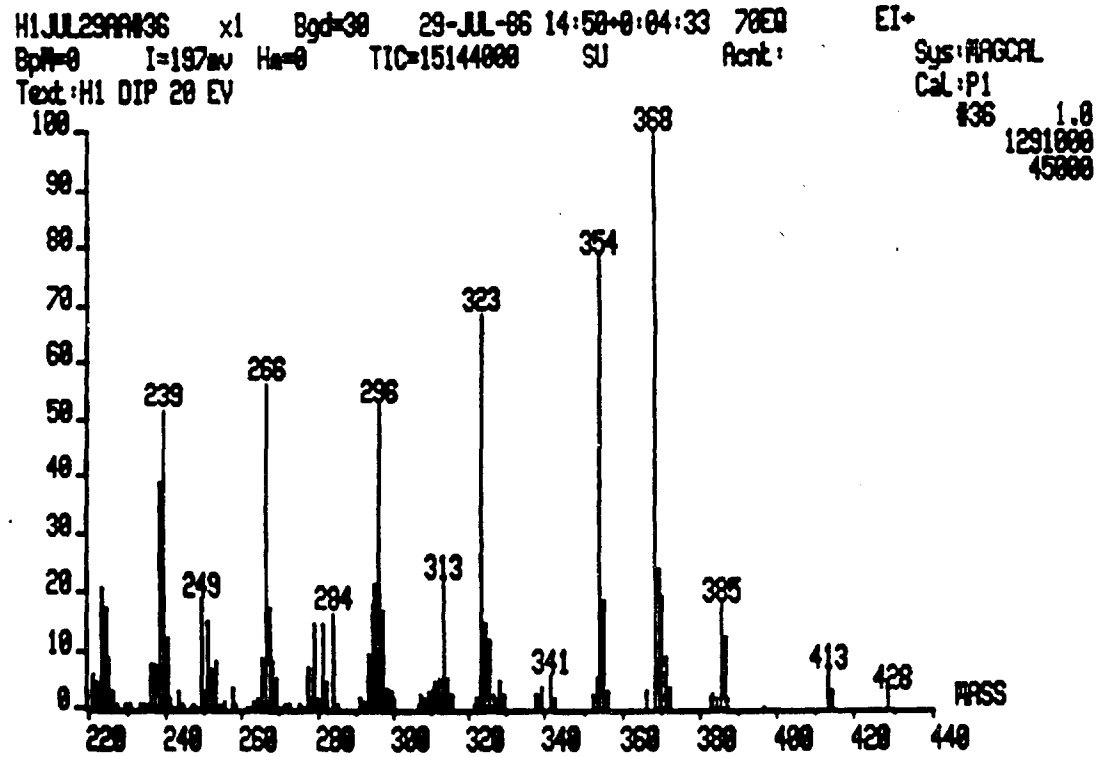


Figure 1. Electron impact.

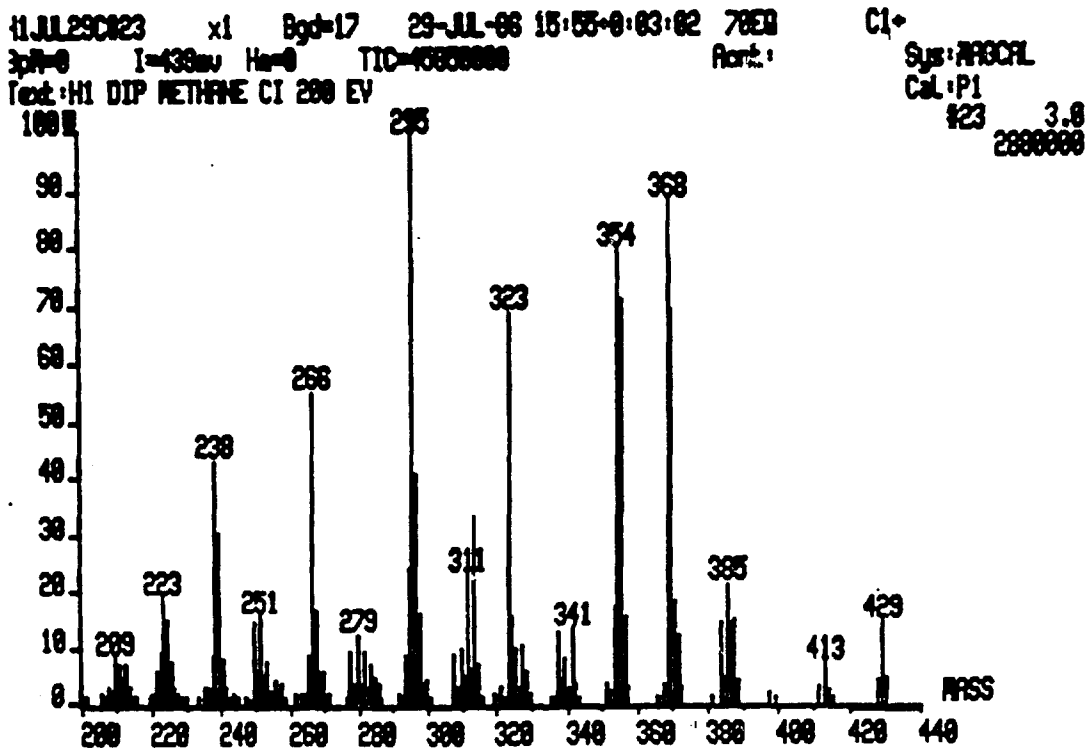


Figure 2. Chemical ionization.

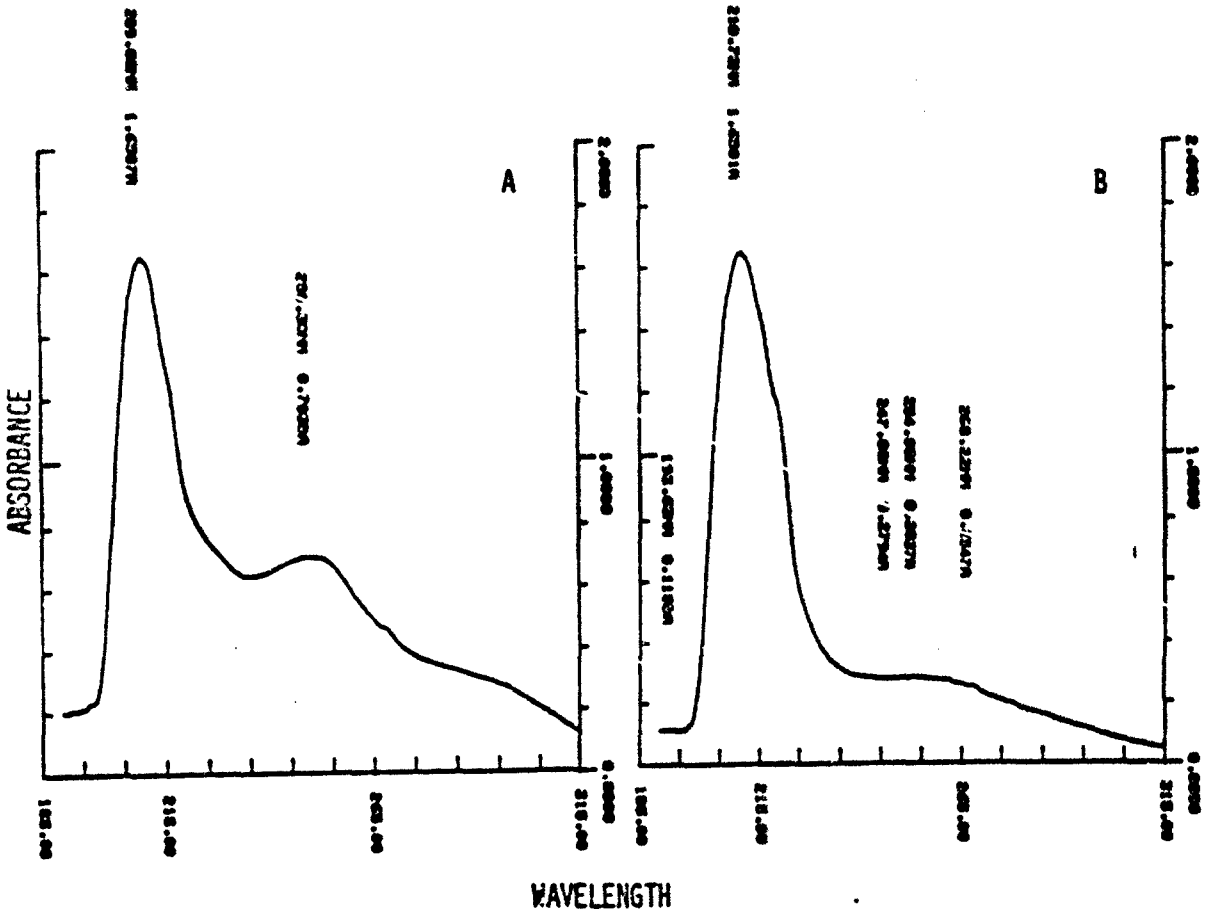


Fig. 3. The UV spectrum of hemorrhagic factor H-1 in purified form (A) and rice inoculated with F. oxysporum N17B extracts (B) in ethanol.

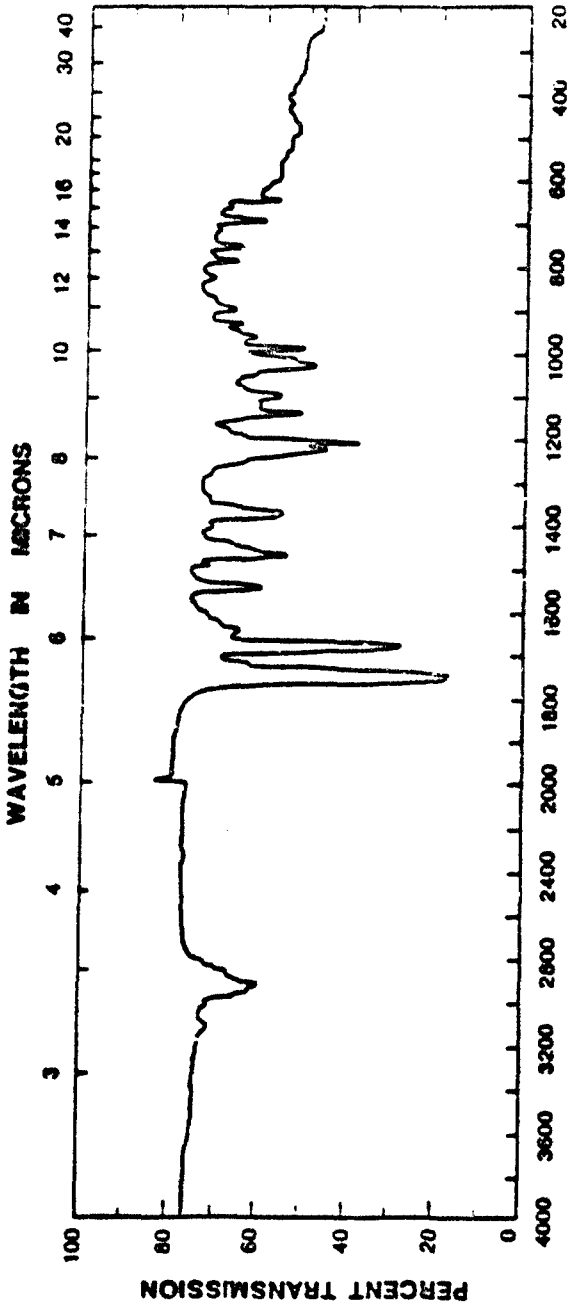


Figure 4. Infrared spectrum of H-1 toxin (KBR pellet).

GALBRAITH
Laboratories, Inc.

QUANTITATIVE MICROANALYSES
ORGANIC – INORGANIC
KNOXVILLE, TENNESSEE 37921

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2323 SYCAMORE DR.

PHONE 546-1335
AREA CODE 615

Mr. Hamed K. Abbas
University of Minnesota
Plant Pathology / 495 Borlaug
1991 Buford Circle
St. Paul, Minnesota 55108

August 25, 1986

Received: August 18th

Dear Mr. Abbas:

Analysis of your compound gave the following results:

Your #,	Our #,	% C,	% H,	% N,	% O,
H-1	P-9950	63.80	6.25	0.11	25.19

The molecular weight results will follow later.

This is confirming our telephone call of August 25, 1986.

Sincerely yours,
GALBRAITH LABORATORIES, INC.

Gail R. Hutchens
Exec. Vice-President

GRH:pd

Pathogenic Studies of H-1 Toxin on Rats

We conducted acute trials with recrystallized H-1 toxin and a subacute trial with H-1 contaminated feed on rats. Acute trials with ethylacetate extracts of contaminated feed were performed on mice and guinea pigs as well.

Trial I: Gavage of Rats with Purified H-2 Toxin.

As a pilot trial for studies with purified toxin, groups of 2 three week, female Sprague Dawley rats were gavaged with a single dose of 50, 25 or 4 mg/kg body weight recrystallized toxin in 0.5 ml 40% ehtanol. Both animals in the 50 mg and 25 mg groups died within 24 hours, as did one of the animals at the 4 mg level. The other animal in the 4 mg group remained clinically normal and was euthanized at 24 hours. Gross lesions included severe hemorrhage of the mucosa of the glandular stomach in one animal of the 50 mg and 4 mg groups, severe cardiac hemorrhage in the rat of the 4 mg group and hyperemia of the duodenum in one rat of the 25 mg group and the rat of the 4 mg group. Histologic evaluation is still in progress, but preliminary results show that purified toxin causes similar lesions to those produced by crude toxin.

Trial II: Gavage of Rats with a Low Dose of Purified Toxin

In an attempt to determine an effective dosage for a future acute study, 9 rats were gavaged with 4 mg/kg recrystallized toxin. Five of 9 died within 24 hours; the remaining animals remained clinically normal and were euthanized at 24 hours. There were no gross lesions.

Trial III: Subacute Feeding Trial in Rats

In order to assess the effect of low level exposure to H-1 toxin, eleven 3 week old, female rats were fed a mixture of 10% contaminated

rice (4 ppm toxin) and 90% complete feed. Food and water intake and body weights were recorded at 48 hour intervals. Packed cell volumes, total serum protein levels, total and differential leukocyte counts were performed on 2 different animals at 48 hour intervals. Bone marrow smears, serum chemistry panels and necropsies were performed at 21 days. Ten controls were fed complete rat diet, ad libitum, for 15 days and 10 controls were fed approximately the same amount of food as was ingested by the treated animals. The trial was ended at 21 days, because several animals died at 20 days.

All treated animals became depressed and diarrheic, but all controls remained clinically healthy. Weight gain and feed conversion data are presented in Table 1. Complete results of serum chemistry, hematology, bone marrow evaluation and histologic examination are still pending, but the treated animals developed greatly elevated blood leukocyte levels.

Table VI: Administration of Crude Extracts to Mice and Guinea Pigs

As a pilot trial to evaluate the comparative toxicity of H-1, ethylacetate extracts of contaminated rice were administered by gavage to 180 g female guinea pigs and by intraperitoneal injection to 3 week, female mice. Groups of 2 guinea pigs were given a single dose of extracts of 10, 20 or 50 g of contaminated rice (44 ppm toxin) in 1-2 ml 20% ethanol. One animal of the 50 g group died within 24 hours with locally extensive gastric hemorrhage. The other animals were euthanized at 24 hours and did not develop clinical or morphologic abnormalities. Histologic examination is pending.

Groups of 3 three week, female mice were dosed with extracts of 0.63 and 1.25 g of contaminated rice. Two of the animals of the 1.25 g

group died within 24 hours, with one showing myocardial hemorrhage and both showing hyperemia of the duodenum. No clinical or gross abnormalities were found in the other mice; histologic examination is still pending.

Comments

These preliminary results indicate that acute administration of purified H-1 toxin results in similar clinico-pathologic effects to those produced by crude toxin. The lack of availability of purified toxin has hampered this portion of the study to some degree, but current production should resolve this problem.

Preliminary results also indicate that H-1 toxin produces lesions in mice and guinea pigs which are similar to those in rats. It also appears that guinea pigs are more resistant to orally administered toxin than rats.

The subacute feeding trial showed high toxicity at a level of approximately 4 ppm. Several animals died on day 20 and the others were very sick when euthanized. We have no explanation for the elevated blood leukocyte counts, but they may be a sequella to the lymphocidal effect noted with the pure toxin.

Our immediate plans are to complete the described studies and to perform more acute studies with purified material. In addition, the low level feeding trial will be repeated and the effects on the humoral response to foreign antigen (sheep red cells) and the *in vitro* lymphocytic response to mitogens will be assessed.

TABLE 1. LC50 VALUES OF THE HEMORRHAGIC FACTOR NAMED H-1 WHEN ADMINISTERED TO RATS , MICE AND GUINEA PIGS.

SPECIES	PURITY OF H-1	AGE DAYS	METHOD OF ADMINISTRATION	LC50 MG/KG	TOXIC SIGNS
RAT	crystals	20	ORAL	4	HI,HS,HT,HH,D
RAT	extract	20	ORAL	3	HB,HI,HS,HT,HH,D
MOUSE	extract	20	I.P.	4	HI,HH,D
GUINEA PIG	extract	20	ORAL	12	HS,D

All animals were purchased from BIO-LAB Corp. St. Paul, MN. Each was intubated or injected with toxin based on the gram equivalents of H-1 found in the toxic culture. Control animals received the same volume of 10, 20 or 40% aqueous ethanol as given to the experimental animals.

TOXICITY LEGEND:

HI= intestinal hemorrhage.
HS= hemorrhage in the stomach.
HT= hemorrhage in the thymus.
HH= hemorrhage in the heart.
D= death.

Table 2. Weight gain and feed consumption of rats given a chronic exposure to H-1 toxin in their diet.*

	Treated		Controls		Pair-fed Controls	
	Day 15	Day 21	Day 15	Day 21	Day 15	Day 21
Average Wt Gain (g)	-18.4	-14.0	57.8	--	56.0	67.4
Wt Gain/ Feed Consumption	-0.2	--	0.5	--	0.5	--

*Rats were given a diet containing 90% nutritionally balanced feed and 10% Fusarium culture containing 4 ppm H-1.

MASS SPECTRAL LIBRARY OF TRICHOTHECENES AND OTHER MYCOTOXINS PRODUCED BY FUSARIUM

I. One of the objectives of this contract is to construct a mass spectral library of trichothecenes so that it could be used in the identification of this group of toxins and also facilitate the study of metabolism of T-2 and other members of this group. A library consisting of approximately 250 mass spectra of trichothecenes was constructed on the Hewlett Packard 5987 Real Time Executer System and delivered to USAMRIID a few years ago. The latter was developed on the Hewlett Packard Quadrupole Mass Spectrometer. It is our intention to transfer these spectra to the VG 7070 system which appears to be a more versatile and efficient system. Moreover, we are also developing a library of the daughter fragments of the trichothecenes on the VG 7070 EQ. The latter is designed to give unequivocal identification of the trichothecenes. We are including a new dimension in this library in so far that it will contain other potent toxins produced by species of Fusarium, i.e. other than the trichothecenes.

IN SUMMARY WE ARE DEVELOPING:
MASS SPECTRAL LIBRARY OF FULL SPECTRA (VG SYSTEM)
MASS SPECTRAL LIBRARY OF DAUGHTER IONS (VG SYSTEM)

A. MASS SPECTRAL LIBRARY OF TRICHOTHECENES (FULL SPECTRA)

An example of our current effort in constructing this library is enclosed. Of more importance, we are showing its uility in identification of a toxic substance produced by Fusarium and present in a biological matrix. An xample of a search follows.

This library is called FUSTOX and presently contains the following spectra:

T-2 toxin
3-hydroxy-4,15,-diacetoxy-8-[3-methyl-butyryloxy]-12,13-epoxy-trichothec-9-ene (TFA)
T2-tetraol (3,4,15-tetrahydroxy-12,13-epoxytrichothec-9-ene(TFA)
HT-2 toxin (3,4-dihydroxy-15-acetoxy-8-[3-methyl-butyryloxy]-12, 13-epoxytrichothec-9-ene (TFA).
Triacetoxyscirpene
8'-hydroxy-zearalenone (alpha isomer)
8'-hydroxy-zearalenone (beta isoamer)
zearalenone (trans)
3'-hydroxy-zearalenone (F-5-2)
H-1 hemorrhagic factor produced by Fusarium (in the process of chemical characterization.
Fusarochromanone (TDP-1) underivatized (causes bone deformation)
Fusarochromanone (TMS ether)
TDP-2 TMS ether. Monoacetyl derivative of fusarochromanone
TDP-2 underivatized
TDP-3 unknown derivative of fusarochromanone(TDP-1)

TDP-4 (Unidentified toxic derivative of Fusarochromanone)
Monoacetyl derivative of Fusarochromanone(synthetic product)

B. USE OF FUSTOX LIBRARY FOR IDENTIFICATION(LIBRARY SEARCH-VG SYSTEM)

An isolate of Fusarium equiseti which originated in Germany was cultured on rice with the intention of determining the presence of Fusarochromanone (Fusarium metabolite that causes tibial dyschondroplasia). A crude extract was obtained and resolved by capillary chromatography on the GC/MS VG-7070EQ. Figure 1 shows the resolution of the TMS derivatives on DB-5 capillary column. Multiple components were found (at least 5) that belonged to this chemical group. Scan 201 was suspected of being Fusarochromanone and was tested by the FUSTOX library. The results of the search of scan 201 is reported in figure 2. Note that among the hits or tentative identifications, entry number 8 is listed as the best fit which agrees with our own interpretation of the spectrum. The library is searched both forward (match the spectrum with the library) and reverse search (match the library with the spectrum). The higher the numbers in the table marked PUR, MIX and REV (respectively 602, 833 and 654) the better the quality of the match. PUR stands for relative purity of the compound, i.e. very few flagged masses; MIX shows the results of the comparison of both the forward and reverse library search and REV shows the results of only the reverse search. A comparison of scan 201 with the library entry is shown in figure 3 ;the spectra are almost identical indicating an excellent implementation of the search system.

Scan #245 of figure 1 was also matched with the library and the results are presented in figure 4. Once more the library correctly identified the FUSAROCHROMANONE derivative (TDP-2). Note the high values of PUR, MIX and REV. The comparison of the library spectrum of TDP-2 with scan 245 is shown in figure 5; the spectra are almost identical. The search routine of the VG USER GENERATED LIBRARY appears to be excellent for implementation for Fusarium toxin identification.

The total ion current chromatogram of the extract from Fusarium is shown in figure 6. The TDP-1 indicator fragment is m/z+ 218 and this ion was used to uncover naturally occurring derivatives of FUSAROCHROMANONE in the culture. At least 8 different derivatives were found. Of the eight, we only identified 2; the remainder are new components.

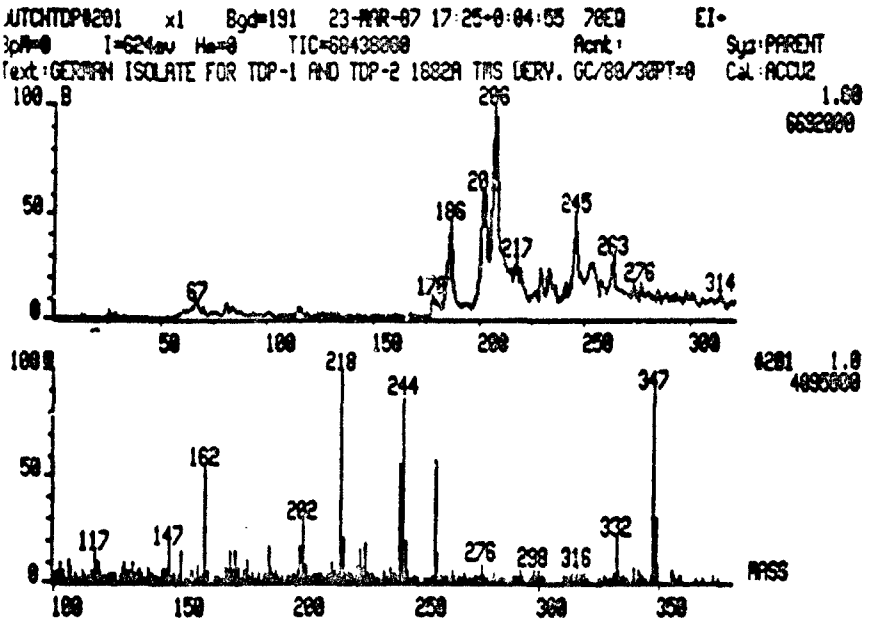


FIGURE 1. TOTAL ION CHROMATOGRAM (UPPER BOX) OF THE CRUDE EXTRACT FROM FUSARIUM EQUISETI OBTAINED FROM GERMANY. THE TMS ETHER DERIVATIVES WERE RESOLVED ON 10 METER CAPILLARY COLUMN (DB-5).(LOWER BOX): MASS SPECTRUM OF SCAN 201 INDICATING THE PRESENCE OF THE TMS ETHER OF FUSAROCHROMANONE (TDP-1).

LIB Library Search Report of DUTCHTDP#201 Library:FUSTOX 23-MAR-87

18 Library spectra compared for BEST FIT
18 matched 8 or more of the 16 largest peaks in the unknown

Fit	Entry	Compound Name	Reference
1	8	TDP-1 (TMS) FUSAROCHROMANONE-TMS 14OCT86 70EQ 70EV EI	0-00-0
2	9	TDP-2(TMS DERIVATIVE) MONOACETYL DER OF TDP-1. 28AUG'8	0-00-0
3	7	TDP-1 (FUSAROCHROMANONE) 70EQ EI 70EV TIC84399000 DIP	0-00-0
4	13	MONOACETYL OF TDP-1 SYNTHESIZED BY XIEGEN. 28AUG'86 70E	0-00-0
5	10	MONOACETYL-TDP-1 OR TDP-2 UNDERIVATIZED 6AUG'86 70EQ 7	0-00-0

Fit	Elements	Bpk	Mwt	pur	MIX	rev
1		347	347	682	833	654
2		218	486	417	561	576
3		218	292	348	600	458
4		218	334	332	571	499
5		162	334	312	589	459

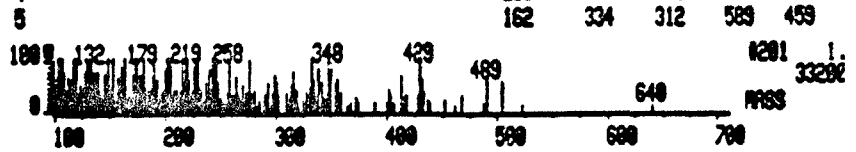


FIGURE 2. REPORT OF A LIBRARY SEARCH MADE ON THE VG SYSTEM OF SCAN 201. NOTE THAT THE BEST FIT IS LISTED AS FUSAROCHROMANONE. THE PURITY IS REPORTED AS 602, RESULTS OF MIXED SEARCH (FORWARD AND REVERSE) IS 833 AND THE REVERSE SEARCH (REV) IS 654. THE HIGHER THE VALUE OF THESE NUMBERS THEN THE BETTER THE CORRELATION OR FIT. MAXIMUM VALUE IS 1000.

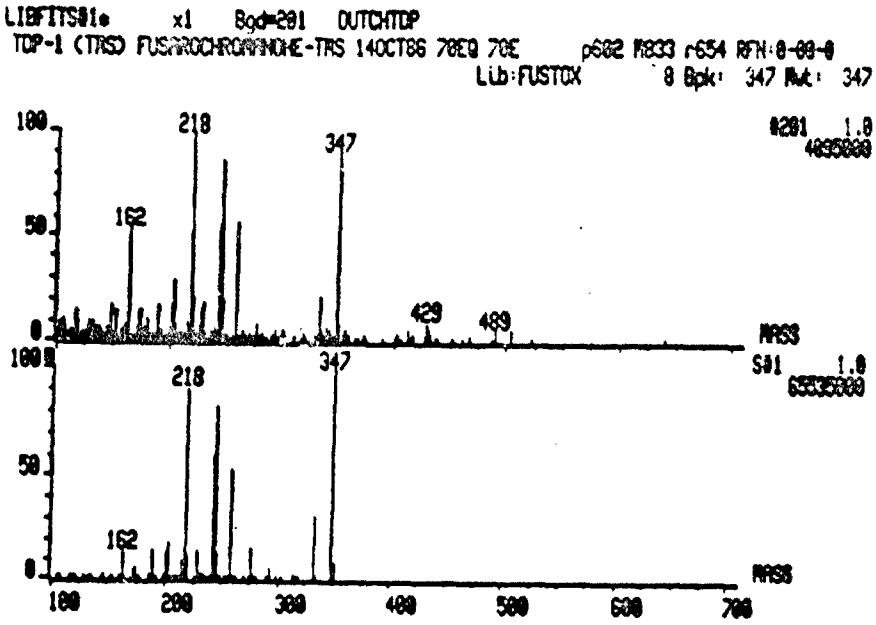


FIGURE 3. COMPARISON OF THE MASS SPECTRA OF THE SCAN 201 (UPPER BOX) WITH THE LIBRARY ENTRY OF FUSAROCHROMANONE. THE SPECTRA ARE ALMOST IDENTICAL INDICATING AN EXCELLENT FIT.

LIB Library Search Report of DUTCHTDP#245 Library:FUSTOX 23-MAR-87

18 Library spectra compared for BEST FIT
18 matched 0 or more of the 16 largest peaks in the unknown

Fit	Entry	Compound Name	Reference
1	9	TDP-2(TMS DERIVATIVE) MONOACETYL DER OF TDP-1, 28AUG'8	0-00-0
2	8	TDP-1 (TMS) FUSAROCHROMANONE-TMS 140CT86 70EV 70EV EI	0-00-0
3	14	TDP-4 (DIACETYL-TDP-1) SYNTHESIZED BY XIEGEH, 3 JUN'86	0-00-0
4	7	TDP-1 (FUSAROCHROMANONE) 70EV EI 70EV TIC043000000 DIP	0-00-0
5	13	MONOACETYL OF TDP-1 SYNTHESIZED BY XIEGEH, 28AUG'86 70E	0-00-0

Fit	Elements	Bpk	Mat	pur	RIX	rev
1		218	406	614	798	725
2		347	347	437	781	484
3		218	376	392	661	453
4		218	292	389	650	492
5		218	334	384	625	546

FIGURE 4. MASS SPECTRAL LIBRARY SEARCH OF THE E.I. SPECTRUM OF SCAN #245 TENTATIVELY SUSPECTED OF BEING TDP-2 (MONOACETYL DERIVATIVE OF FUSAROCHROMANONE). TDP-2 WAS SELECTED CORRECTLY AS THE BEST FIT ; THE PURITY, MIXED SEARCH AND REVERSE SEARCH VALUES WERE HIGH INDICATING A GOOD MATCH.

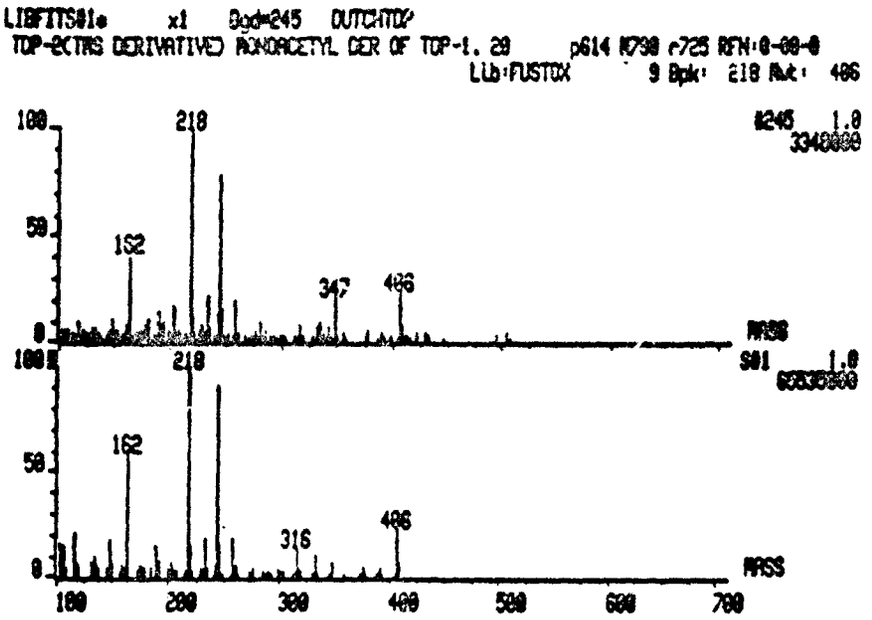


FIGURE 5. COMPARISON OF THE MASS SPECTRA OF SCAN #245 OF THE CRUDE EXTRACT AND THE LIBRARY COPY OF THE TDP-2 STANDARD. CASUAL INSPECTION WOULD INDICATE THAT THE SPECTRA ARE ALMOST IDENTICAL.

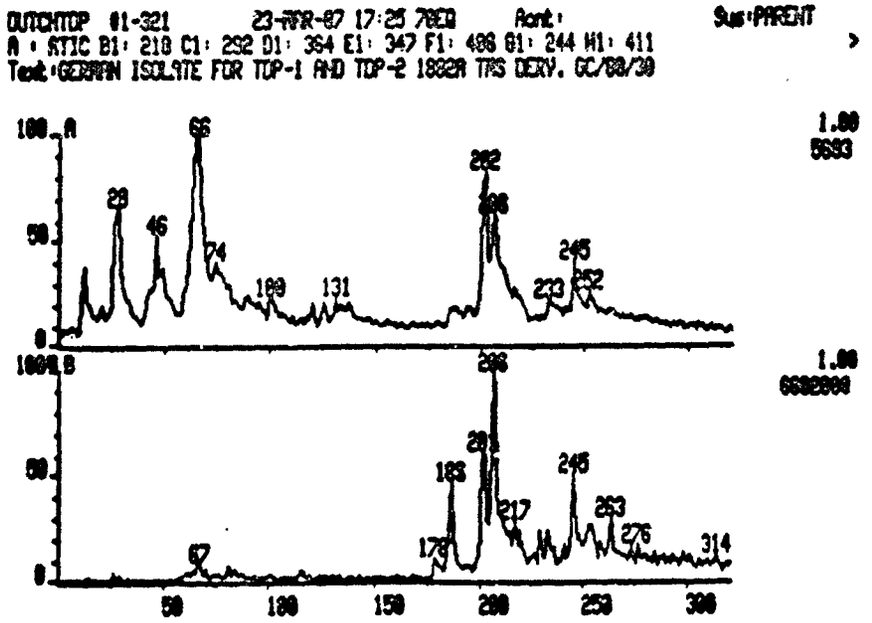


FIGURE 6. THE TOTAL ION CHROMATOGRAM OF THE CRUDE EXTRACT OF THE FUSARIUM CULTURE IS SHOWN IN BOX A. BOX B SHOWS THE TOTAL ION CHROMATOGRAM OF MASS 218 WHICH IS THE BASE PEAK OF TDP-1 AND AN EXCELLENT INDICATOR OF THE TDP-1 NUCLEUS. THE TRACING IN BOX B INDICATES THAT AT LEAST 7 DIFFERENT DERIVATIVES OF FUSAROCHROMANONE ARE PRESENT IN THIS MIXTURE. SCANS 186, 201, 202, AND 245 ARE MOST ABUNDANT. SCANS 201 AND 245 ARE TDP-1 AND TDP-2 RESPECTIVELY; THE OTHER COMPONENTS HAVE NOT BEEN IDENTIFIED.

INTERFERING SUBSTANCE IN HUMAN BLOOD AND ITS ANALYSIS BY THE DAUGHTER ION MASS SPECTRAL LIBRARY

During the course of analysis of human urine for various metabolites of T-2 toxin, it came to our attention that a substance present in control blood was interfering with our analysis of T-2 toxin by Multiple Reaction Monitoring (MRM). The material did not actually interfere with T-2 because it had a different retention time on the capillary column but theoretically it was not supposed to pass through the magnet. By way of background, in MRM analysis of T-2(TFA), the magnet is parked on m/z+ 478 and the quadrupole is scanning for selected daughter ions (the quadrupole is programmed to detect only the legitimate daughters of the compound in question). This means that either the daughters are there or they are not; the quadrupole will not detect alien daughter fragments. The magnet end is set on the accurate mass of 478.14506 to insure that it sits directly on top of the selected parent ; this does not mean that because of accurate mass selection that the instrument is scanning at high resolution; it is not. Normally we operate at about a resolution of 1000. One does not expect any appreciable noise to filter through the system. However, in the analysis of blood, another 478 mass fragment appeared at a retention time of about 30 seconds past that of T-2 (figure 7-A). The peak was appreciable and at low concentrations of T-2, caused the T-2 peak to look insignificant because of its normalization to the most abundant peak. We decided to analyze the interfering peak with the daughter ion library and to compare it with daughters of T-2-TFA.

The total ion chromatogram of the interfering substance is shown in figure 7. The peak is shown in scan 134 and its daughter ions are shown in the lower box. We could not detect any m/z+ 478 fragment in the daughter ion scan and could not detect any fragments characteristic of T-2 -TFA daughters. As shown in figure 8, the DAUGHTER ion mass spectral library did not generate any match for the interfering blood substance. In contrast, a sample containing T-2 toxin was run in the daughters system and its daughter ion spectrum was matched with the library. As noted in figure 9, a good fit was found in the library entry and all the daughter ions were found. A comparison of the library entry with that of the unknown is shown in figure 10. Note the identical daughter ion spectrum of mass 478 of the library and the sample. The parameters of the library scan i.e. values used in directing the search, are shown in figure 11. It is important to adjust the fit threshold (THR) to 100 or lower.It is also helpful to enable the filters to select only those samples that have the correct daughters.

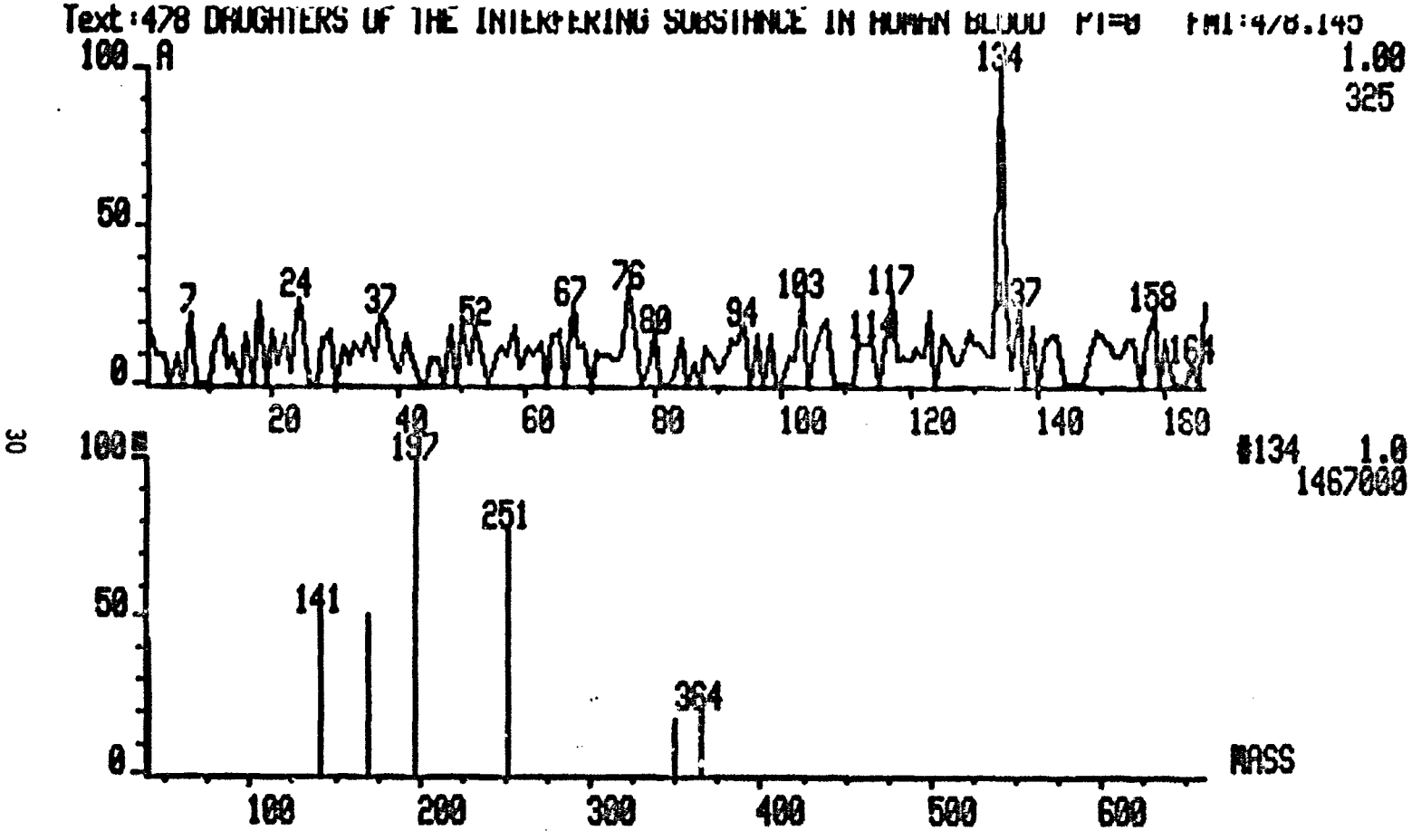


FIGURE 7. DAUGHTER ION SPECTRUM OF AN INTERFERING SUBSTANCE PRESENT IN CONTROL HUMAN BLOOD WITH A M/Z+ 478 FRAGMENT SIMILAR TO THAT OF T-2-TFA. NOTE THAT THE 478 FRAGMENT IS RAPIDLY DECOMPOSED AS NO TRACE OF IT IS FOUND ONCE IT LEFT THE COLLISION CHAMBER (CAD) AND THAT THE DAUGHTERS ARE DIFFERENT FROM THOSE FOUND IN T2-TFA. THE MAGNET WAS PARKED ON MASS 478.145 ANMD THE QUADRUPOLE WAS SCANNING BETWEEN MASSES 700 AND 50. THE RETENTION TIME OF T-2 TFA IS ABOUT 6.2 (EQUIVALENT TO SCAN 117) AND ITS ANALYSIS IS NOT INFLUENCED BY THE UNKNOWN SUBSTANCE SHOWN ABOVE. HOWEVER, THE SUBSTANCE IS DESCRIBED BECAUSE CONTRARY TO EXPECTATION, IT HAS AN IDENTICAL MASS OF 478.

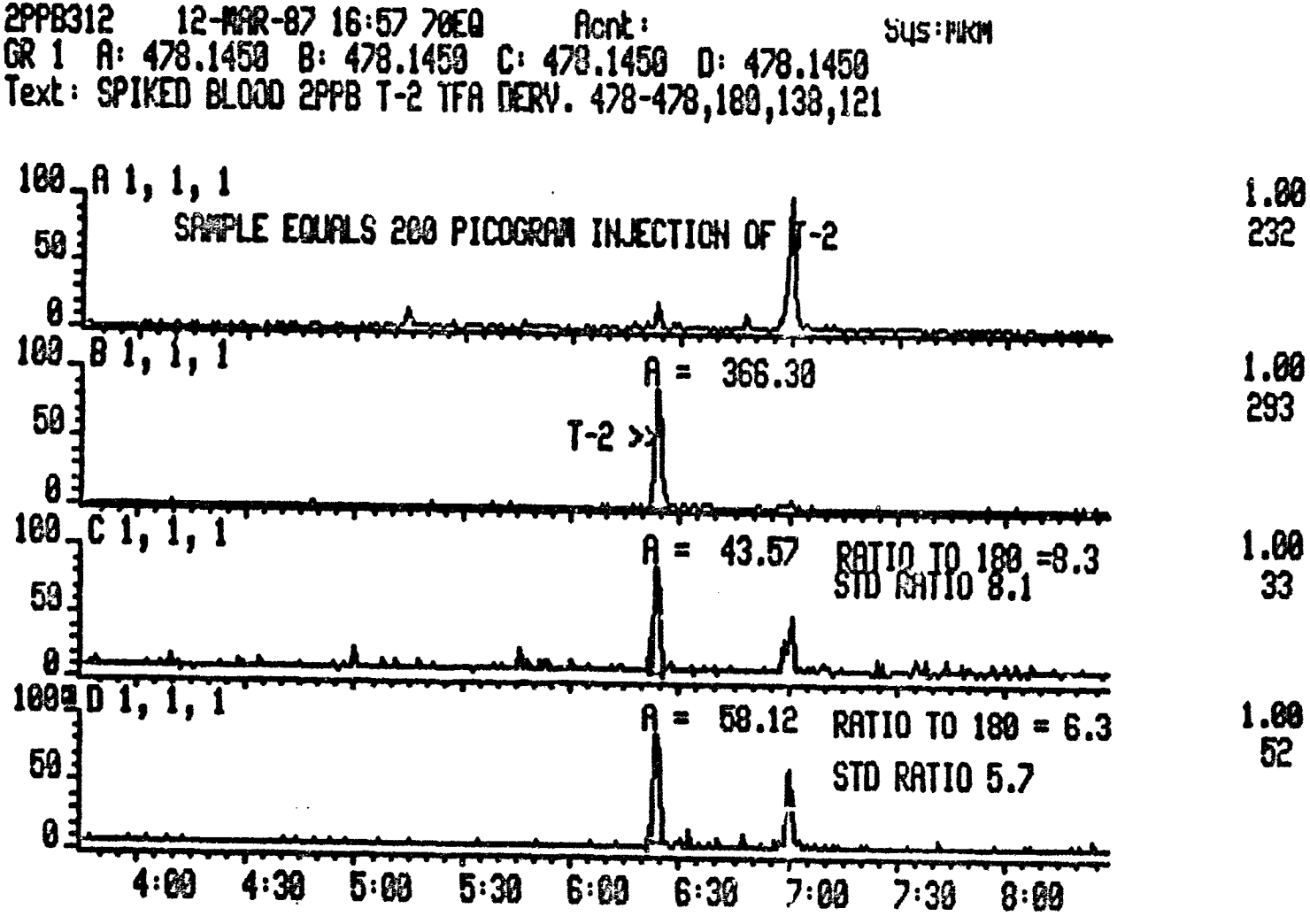


FIGURE 7A. Resolution of the T-2 toxin TFA from the interfering substance found at retention time 7.0 whereas T-2-TFA is found at 6:25. Note that the T-2 concentration is equivalent to 200 picograms which in box A (fragment 478 is monitored) is actually in a concentration higher than T-2. However, boxes B, C and D are true daughters of T-2 (180, 138 and 121 respectively) and no problem was encountered in detection.

LIB LLibrary Search Report of BLDSUB#134 LLibrary:DAUGHTER 13-MAR-87

6 LLibrary spectra compared for BEST FIT
6 matched 0 or more of the 16 Largest peaks in the unknown

Flt	Entry	Compound Name	Reference
NO FITS FOUND			

Flt	Elements	Bpk	Mwt	pur	MIX	rev

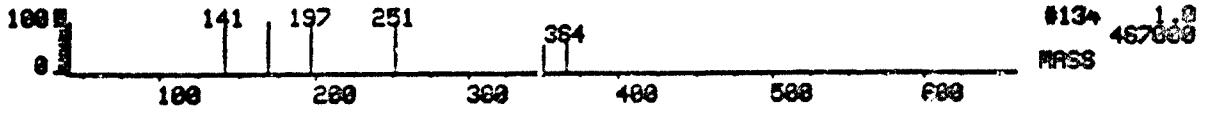


FIGURE 8. RESULTS OF A LIBRARY SEARCH OF THE DAUGHTER ION MASS SPECTRAL LIBRARY OF TRICHOTHECENES. NO FIT OR MATCH WAS FOUND BECAUSE THE DAUGHTERS OF THE UNKNOWN DID NOT CORRESPOND TO THOSE OF T-2-TFA. THIS SEARCH SERVES AS A TEST TO PROVE THAT ALTHOUGH AN UNKOWN MAY HAVE A FRAGMENT SIMILAR TO T-2-TFA, IT CAN BE EASILY BE DISCRIMINATED FROM T-2-TFA BY MEANS OF CHARACTERISTIC FRAGMENT IONS. F

LIB LLibrary Search Report of 478T2876 LLibrary:DAUGHTER 13-MAR-87

6 LLibrary spectra compared for BEST FIT
6 matched 0 or more of the 16 Largest peaks in the unknown

Flt	Entry	Compound Name	Reference
1	5	DAUGHTERS OF 478 OF TFA T-2. 70EQ 70EV EI TIC 68[illegible] 300	0-00-0
2	1	DAUGHTERS OF 400 OF T2-TFA. 29JUL'86 70EQ 70EV EI TICS	0-00-0

Flt	Elements	Bpk	Mwt	pur	MIX	rev
1		121	0	360	496	548
2		400	0	12	97	101

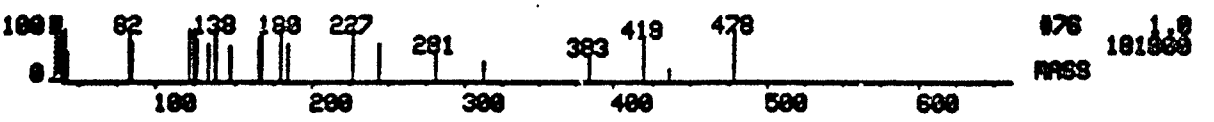


FIGURE 9. RESULTS OF THE LIBRARY SEARCH OF THE DAUGHTER ION MASS SPECTRAL LIBRARY FOR T-2-TFA. THE LATTER WAS DETECTED IN A URINE SAMPLE AND ITS IDENTITY VERIFIED BY MEANS OF THE LIBRARY. NOTE THE HIGH VALUES IN THE MIX AND REV PARAMETERS WHICH INDICATE A HIGH PERCENTAGE PROBABILITY OF IDENTIFICATION OF THE T-2-TFA.

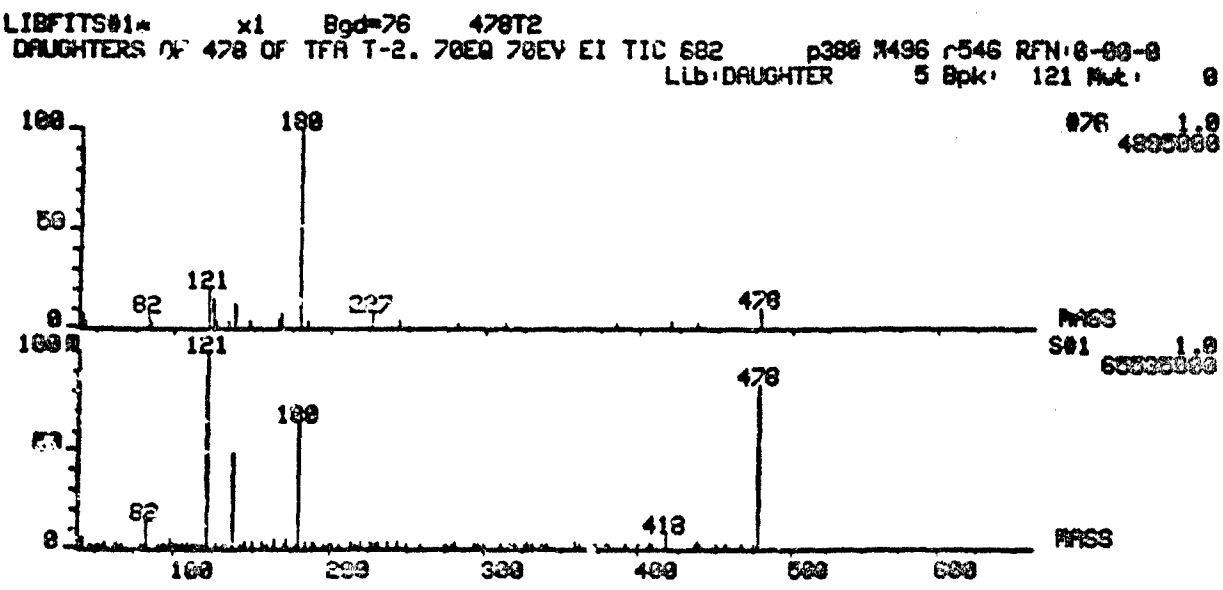


FIGURE 10. COMPARISON OF THE DAUGHTER ION LIBRARY SPECTRUM OF T-2-TFA WITH THE UNKNOWN FOUND IN THE URINE SAMPLE SHOWN IN FIGURE 9. THE COMMAND "SH" ACTIVATES THIS COMPARISON. NOTE THE IDENTICAL MASSES IN EACH. THE INTENSITIES OF FRAGEMENTS 478, 180 AND 121 VARY BECAUSE OF THE DIFFERENCE IN COLLISION ENERGY USED IN EACH DETERMINATION. IT IS ACCEPTED THAT COLLISION ENERGY WILL VARY AND THUS THE ABUNDANCE OF DAUGHTERS IONS WILL BE AFFECTED; HOWEVER, THIS DOES NOT APPEAR TO BE SERIOUS ENOUGH TO INFLUENCE THE LIBRARY SEARCH.

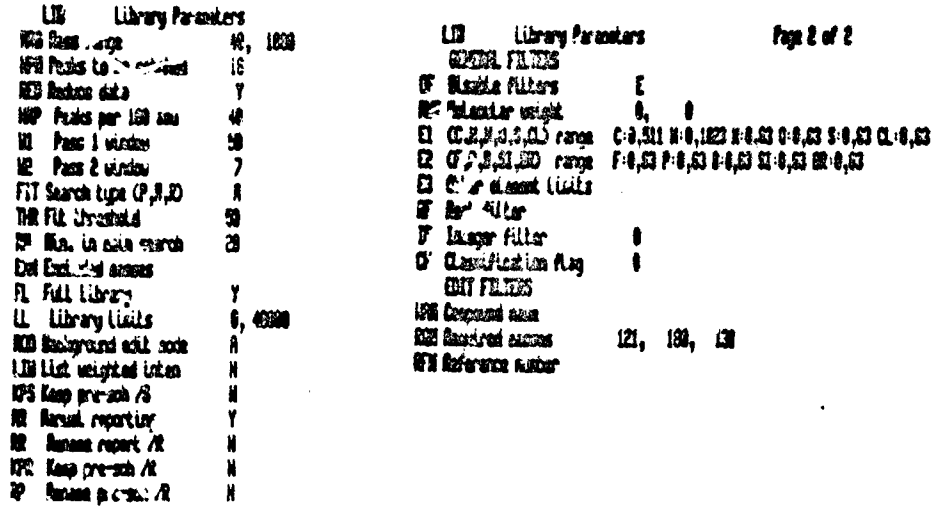


FIGURE 11. PARAMETERS USED IN THE LIBRARY SEARCH ROUTINES WHICH ALLOW VERSATILITY IN A SEARCH. AS AN EXAMPLE, THE PARAMETER "RQM" CAN BE ADJUSTED SO THAT REQUIRED DAUGHTERS MUST BE PRESENT IN THE SPECTRUM BEFORE THE SEARCH ROUTINE WILL CONSIDER A MATCH. IN THIS EXAMPLE, M/Z+ 121, 180, AND 138 WERE SELECTED AS MASS FILTERS. THE PARAMETER (DF) MUST BE ENABLED (ENABLE FILTERS) BEFORE DISCRIMINATION WILL BE ACTIVATED. ACCORDINGLY THE FILTERS CAN BE DISABLED BY TYPING IN "D". THE FIT THRESHOLD "THR" TIGHTENS THE SEARCH PARAMETERS BY CAUSING A GREATER DEGREE OF SELECTIVITY AS ITS VALUE IS DECREASED. IN THIS EXAMPLE, A THR VALUE OF 50 WAS SELECTED.

ANALYSIS OF T-2-TFA USING MULTIPLE REACTION MONITORING

The use of multiple reaction monitoring (MRM) for trace analysis of T-2 toxin is based on prior experimentation in which appropriate parent ions are studied and selected for use in the analytical system. As an example, the daughters of T-2-TFA were determined and then selected for ultimate sensitivity in the assay. Thus, as one examines the full spectrum of T-2-TFA in E.I.(figure 12) it suggests that certain fragments be studied. The molecular ion (562) is normally too weak to be seen but after magnification(5X), it is readily visible. Fragments such as m/z+461, 478, 401 and 327 appear significant enough to yield acceptable analytical results. Figure 13 shows the daughter ions of m/z+ 401 and careful inspection indicates that although the parent ion is appreciable, the daughters are very weak. In contrast, the daughters of m/z+478 give very intense fragments and have all the characteristics of a good ion to monitor i.e. strong daughters in the low mass region and low signal to noise ratio (figure 14). The fragments of interest are: 478, 180, 138 and 121. These are then used qualitatively and quantitatively in subsequent tests for the analysis of trace (2ppb) of T-2-TFA in the biological matrix of blood.

Human blood samples were amended with the equivalent of 2ppb of T-2 toxin and extracted and cleaned by our general laboratory procedure. The extract contained a biological matrix ; the trifluoracetate derivative of T-2 was made in situ and injected into the VG GC/MS operating in the MRM mode. The calculated amount injected into the capillary column was approximately 160 picograms after accounting for a 20% loss in extraction. Note the excellent sensitivity and base line when the m/z+ 180 daughter is monitored (figure 15. The analysis and quantitation of daughters 180, 478, 138 and 121 are shown in figure 16. The multiple reaction monitoring system of analysis appears to be the best yet developed in our laboratory for T-2 toxin analysis. The most important determinant in the analysis is the lack of spurious peaks and electronic noise when working at this level. Our signal to noise ration at 2 parts per billion is about 100:1, the best we have ever achieved. We recommend this method of analysis because of its sensitivity and unequivocal identification of T-2 toxin.

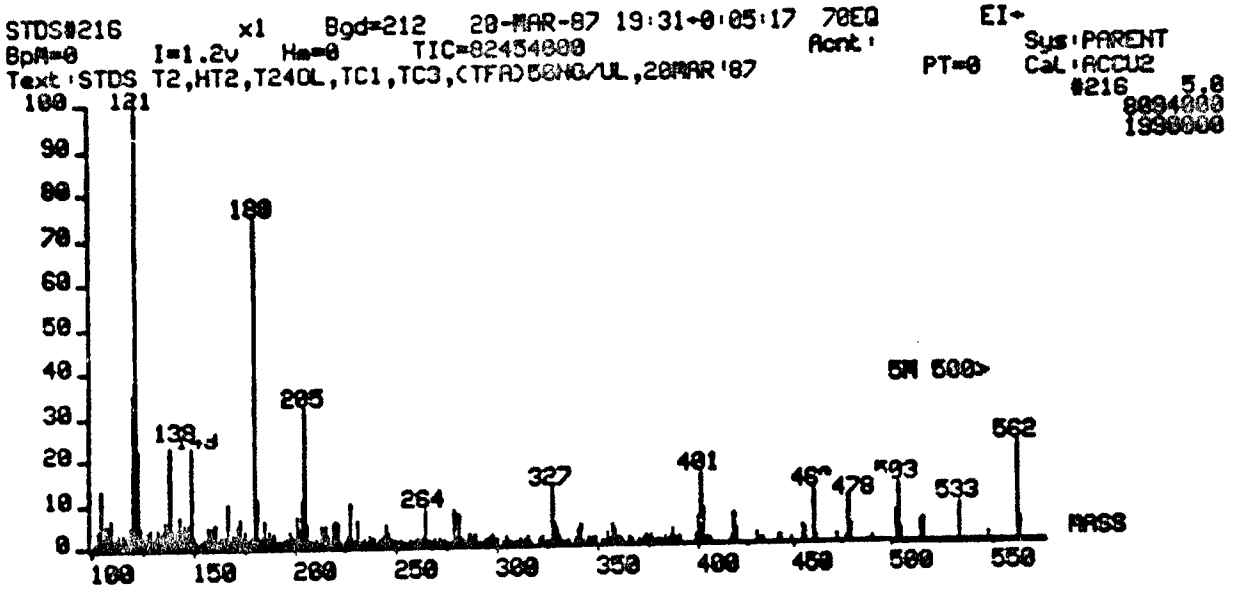


FIGURE 12. FULL SCAN MASS SPECTRUM OF T-2 -TFA IN E.I. AT 70 EV AND A SOURCE TEMPERATURE OF 150 C. NOTE THE MOLECULAR ION (562) WHICH NORMALLY IS NOT VISIBLE. WE USE A SOURCE TEMPERATURE OF 200 TO ACHIEVE THESE REPRODUCABLE RESULTS. THE FRAGMENTS OF INTEREST ARE 478, 460, 401 AND 327.

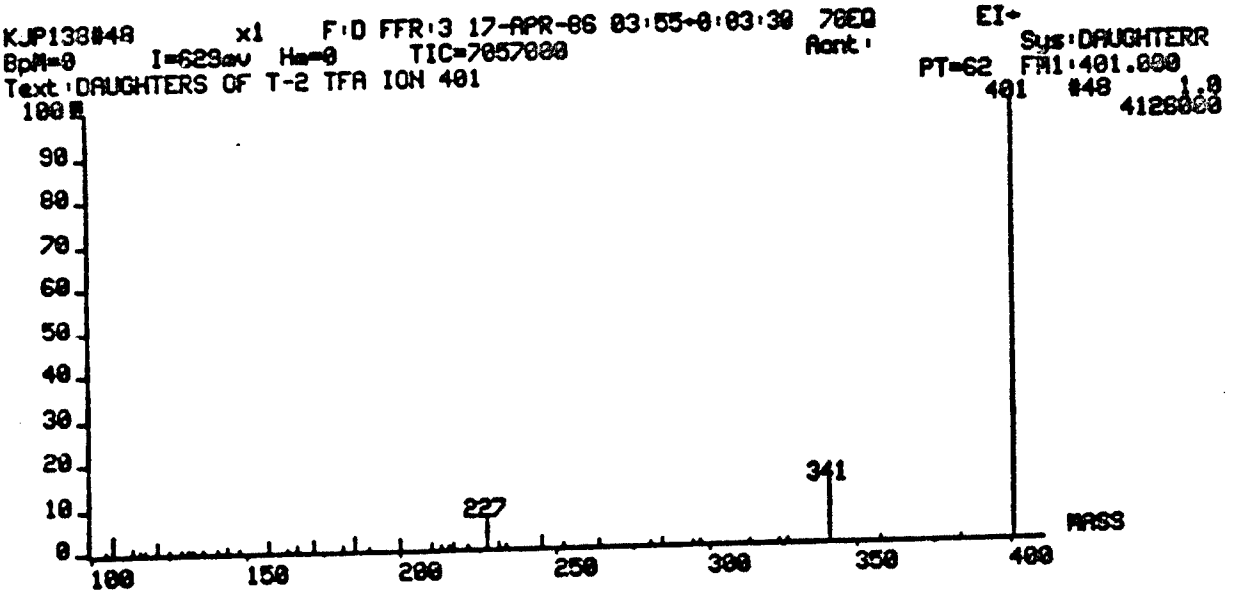


FIGURE 13. DAUGHTER IONS OF FRAGMENT 401 . NOTICE THAT VERY FEW FRAGMENTS OF INTEREST ARE GENERATED AND ALL ARE OF LOW INTENSITY.

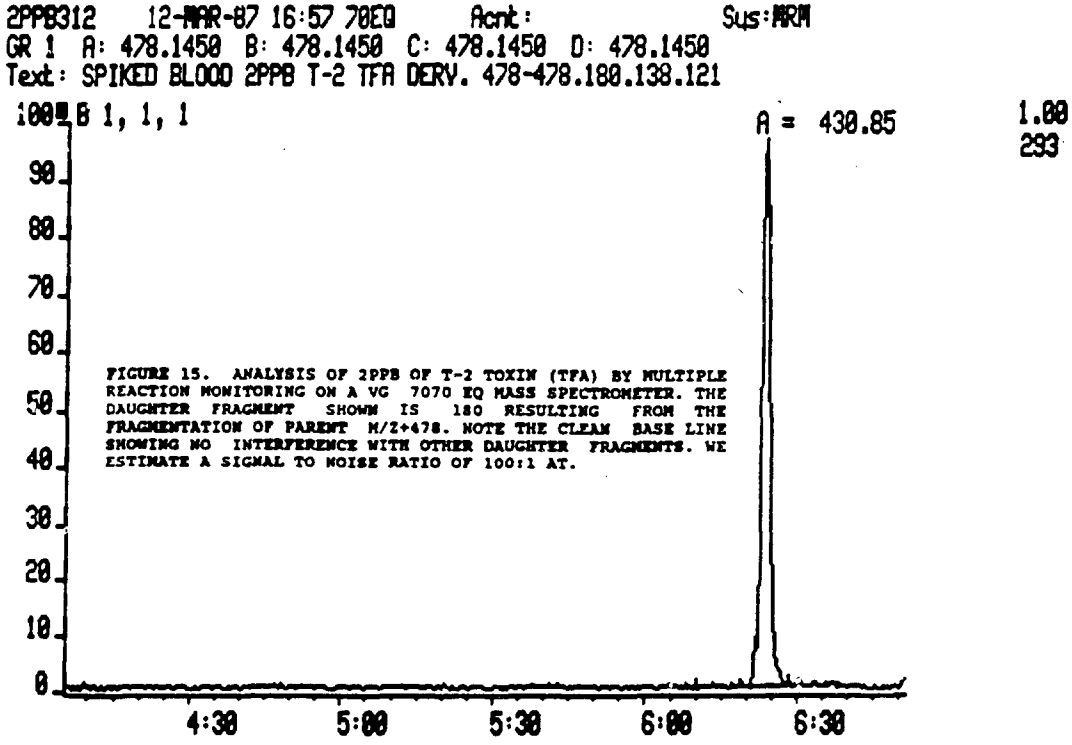


FIGURE 15. ANALYSIS OF 2PPB OF T-2 TOXIN (TFA) BY MULTIPLE REACTION MONITORING ON A VG 7070 EQ MASS SPECTROMETER. THE DAUGHTER FRAGMENT SHOWN IS 180 RESULTING FROM THE FRAGMENTATION OF PARENT M/Z+478. NOTE THE CLEAN BASE LINE SHOWING NO INTERFERENCE WITH OTHER DAUGHTER FRAGMENTS. WE ESTIMATE A SIGNAL TO NOISE RATIO OF 100:1 AT.

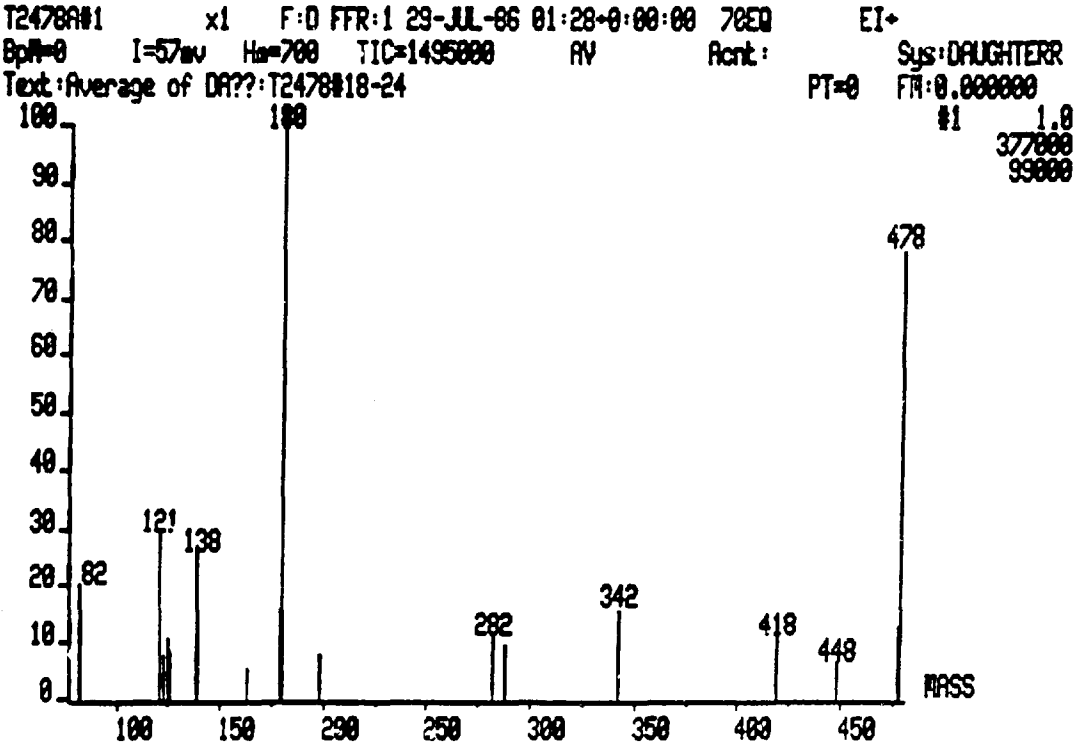


FIGURE 14. DAUGHTER ION OF FRAGMENT 478. THE DAUGHTER SPECTRUM IS RICH IN INTENSE FRAGMENTS ESPECIALLY 180, 138 AND 121. WE HAVE SELECTED THE PARENT 478 AND ITS DAUGHTERS 180, 138 AND 121 FOR MONITORING. THESE FRAGMENTS GIVE US MAXIMUM SENSITIVITY IN A BLOOD MATRIX (1PPB) AND A VERY LOW SIGNAL TO NOISE RATIO AS WILL BE SEEN IN SUBSEQUENT FIGURES.

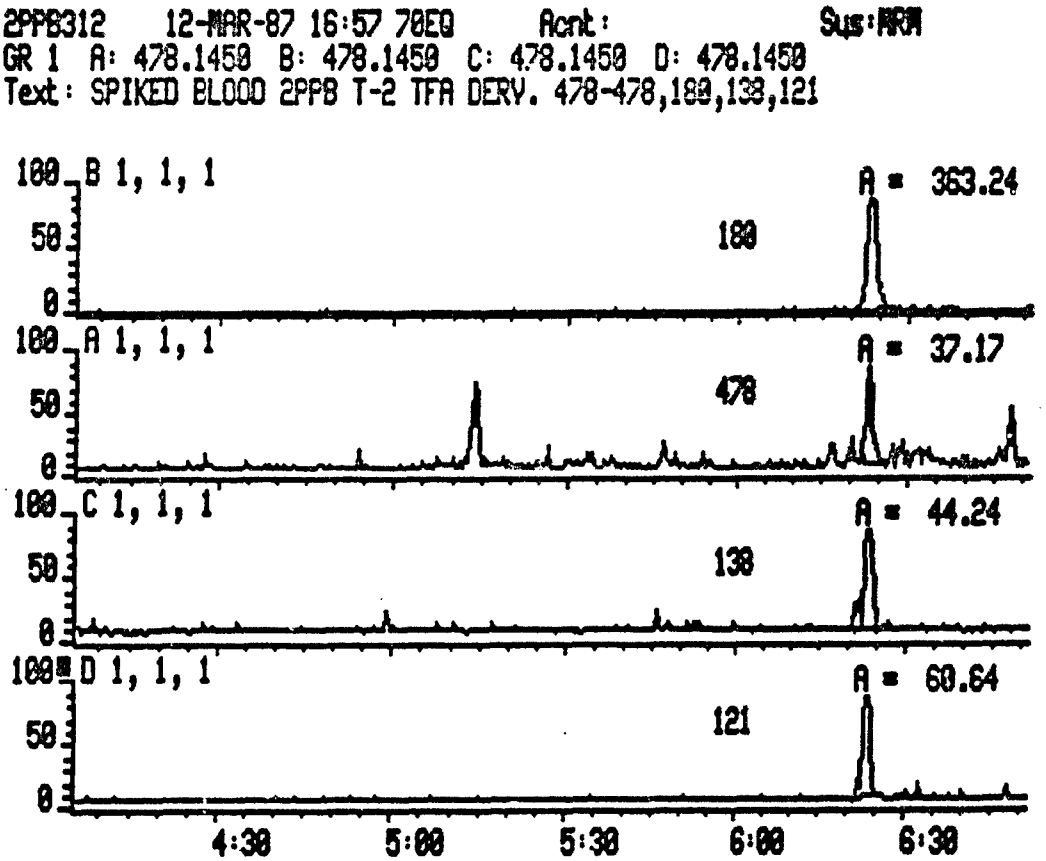


FIGURE 16. ANALYSIS OF 2PPB OF T-2 TFA IN HUMAN BLOOD USING MULTIPLE REACTION MONITORING. THE PARENT FRAGMENT USED IS 478 AND THE DAUGHTERS MONITORED ARE 478, 180, 138 AND 121.

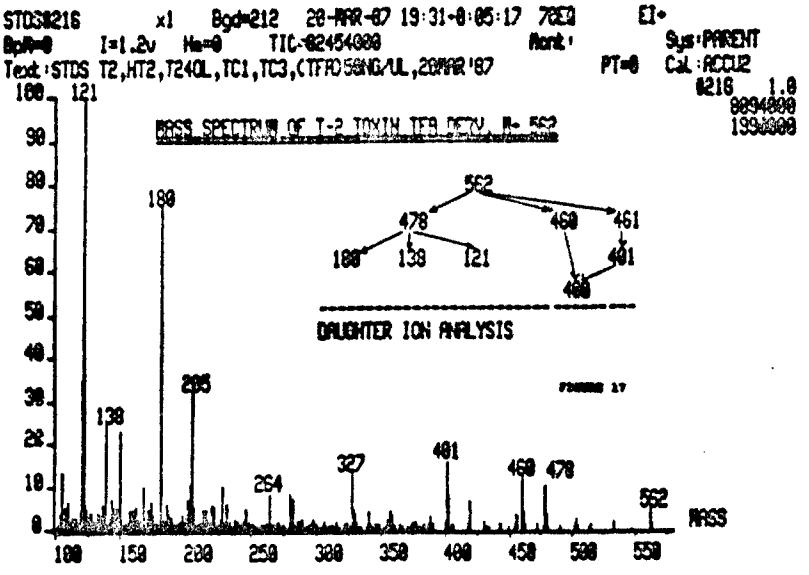
DAUGHTER IONS OF THE TFA DERIVATIVES OF T-2, HT-2 AND T-2-TETRAOL

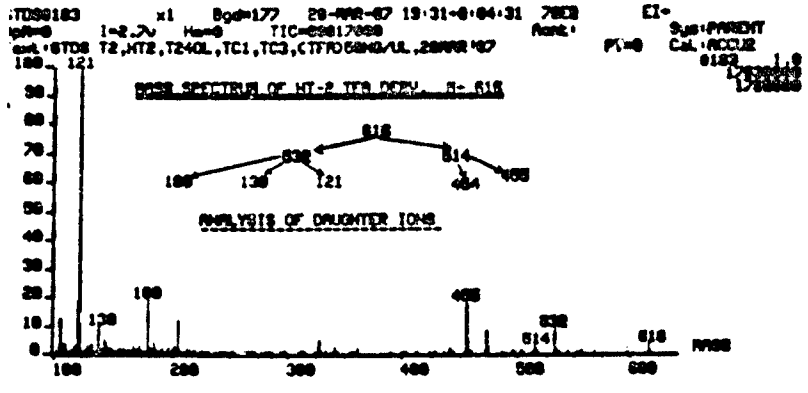
Monkey urine samples were received during tha first quarter of 1987 with the intention of analyzing for T-2 toxin and its derivatives during various times after dosage of the animals. In order to accomplish this task, it was necessary to first determine the daughter ions of some of the expected derivatives in order to analyze by multiple reaction monitoring. This task has been accomplished for T-2 , HT-2 and T2-tetraol. The daughters are shown in the following figures.

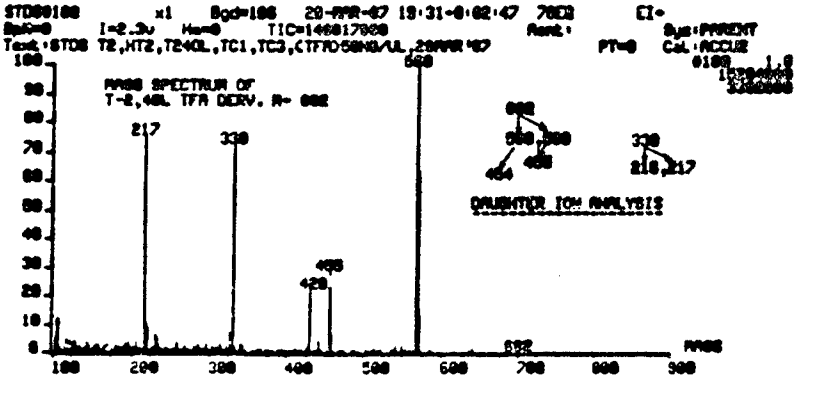
Figure 17 shows the full mass spectrum of T-2-TFA and the daughters of the major ions. Note that the molecular ion 562 gives rise to 478, 460 and 461. The daughter 400 is reached by multiple reactions from 460 and 401. The parent of choice in the MRM analysis is 478.

Figure 18 shows the full mass spectrum in E.I. of HT-2 toxin (TFA). The molecular ion 616 fragments to 532 which in turns gives rise to 180, 138, and 121 as in T-2 except that the origin of these daughters in T-2 is from 478. The parent ion 532 is the fragment of choice for MRM analysis.

Figure 19 shows the full mass spectrum of the TFA derivative of T-2 -tetraol. The molecular ion is seen in E.I. but is not very intense although if detected it gives rise to very intense daughters. Parents 568 and 569 give rise respectively to daughters 454 and 455. Parent 330 is very intense and gives rise an intense 217 daughter and is the choice in the analysis of T-2-tetraol by MRM.


MASS SPECTRUM OF T-2 TOXIN TFA DERV. M= 562
DAUGHTER ION ANALYSIS
MASS


MASS SPECTRUM OF HT-2 TFA DERV. M= 616
ANALYSIS OF DAUGHTER IONS
MASS


MASS SPECTRUM OF
T-2,4BL TFA DERV. M= 668
DAUGHTER ION ANALYSIS
MASS

ANALYSIS OF T-2 TOXIN IN HUMAN BLOOD USING TANDEM MASS SPECTROMETRY GAS CHROMATOGRAPHY

INTRODUCTION

The utilization of combined gas chromatography tandem mass spectrometry for the analysis of T-2 toxin in blood is presented here. The analysis was carried out to confirm and quantitate the presence of T-2 toxin at a low part per billion level (2ppb in 2mL blood). The procedure involved spiking fresh whole blood with the toxin, extraction with an organic solvent, derivatization with trifluoroacetic acid anhydride, and GC/MS/MS analysis. Please remember that the following work was carried out in a biological matrix consisting of the equivalent of 2ml of human blood.

AMENDING BLOOD WITH T-2 AND EXTRACTION

Blood samples (2mL) were amended with T-2 (1ng/ul) in methanol. They were centrifuged for 2 minutes at 7000 rpm in 30 mL tubes with 0.5 mL KH_2PO_4 and 10 mL of toluene:acetonitrile (95:5 v:v). The organic layer was pipetted into a round bottom flask and evaporated on a rotary evaporator. Samples were transferred to half dram vials with methanol and evaporated under nitrogen with gentle heat.

DERIVATIZATION

The dried down samples were derivatized with trifluoroacetic acid anhydride (100 uL) and heated to 60 degrees C. for 20 minutes. After which the excess reagent was evaporated under nitrogen. Samples were brought up in 20 uL of toluene for injection. 1 ul was taken for injection.

GC CONDITIONS

Samples were injected onto a 10 meter fused silica capillary column via a Hewlett Packard on column injection device. The injection device is shown in figure 1. The oven temperature was programmed from 80 to 300 at 30 deg. per second. T-2 eluted at 6.28 minutes into the run. The helium gas flow was set to 10 psi.

In addition to the on column injection technique a 2 ppb blood sample was prepared for splitless injection. The technique afforded overall better resolution and narrowly defined peaks. Signal to noise ratios for 2ppb samples were better than 14 to 1. This method proved superior to the present on column technique. Injecton was done on a 10 meter capillary column. Column purge was activated at 0.6 minutes after injection.

SOURCE CONDITIONS

A standard VG 7070 EI/CI source was used in the analysis. The instrument was operated at 6kV, 70 electron volts. Source temperature was set at 100 degrees. Resolution was set to 2000.

MS/MS TUNING

The instrument was tuned for MS/MS analysis with perfluorotributyl amine. Intermediate and quad lens settings were set to allow maximum transmission of parent ion 614. The quadrupole mass analyzer was calibrated with the VG software LOW MASS (LM) and HIGH MASS (HM) ion commands. The high resolut portion of the mass spectrometer was tuned with perfluorokerosene using the CAL programs.

COLLISION ACTIVATED DECOMPOSITION (CAD) set-up:

Argon was used as the collision gas and the chamber was pressurized to 1 X 10 -6 torr. Collision energy was set at 15 volts.

FA3 AND MULTIPLIER SE :

The gain was set on FA3 unit to 2 X 10-7. Response time was set to 0.01 and multiplier set to 2.10.

MULTIPLE REACTION MONITORING PROGRAM (MRM):

Selected Ion Recording (SIR) was called up and MRM was set up to monitor the decomposition products (daughters)of ion 478 from T-2 TFA, to 180, 138 and 121 as seen in the figures. The exact mass 478.145 was used to set the parent ion correctly. When running the program we found it better to set the magnet manually by positioning it over the parent ion rather than letting the data system do this. To do this one must allow the data system to have control over quad scanning only. The 7070EQ is equipped with knobs on the scan control unit to select this type of scanning procedure. The parameter settings are outlined in figure 4.

RESULTS:

The electron impact mass spectrum is shown in figure 2. Please note that the molecular ion (M+=562) is visible in the spectrum. This was accomplished by lowering the source temperature to 100 degrees C. This is not the normal procedure in EI as the normal operating temperature is 200 C. Our source heater was not operative in this situation and this allowed us to reach the lower temperature setting. We are not recommending this temperature setting. A typical ON-COLUMN injection resolution is found in figure 5. The ions monitored were 180, 138 and 121 and their ratios as daughters are shown. The ratios were satisfactory however the resolution of the components of the standard did not meet out criteria of acceptability. The peak sensitivity and peak width was not acceptable. Figure 5 shows resolution of T-2 TFA as the pure

standard whereas figure 6 shows T-2-TFA in a biological matrix.

The parent ion 478 gave distinct and intense daughter ions that (figure 3) allowed elimination of other components from the background except for one present in human blood and having the exact mass (478) as T-2-TFA toxin. This metabolite (figures 7and 8) found in these blood samples did not interfere with the analysis of T-2 because it resolved itself nearly one minute from T-2. In addition, the ion ratios for the interference did not fit the T-2 toxin and therefore would not be confused with T-2(figure 7). However, it is mysterious that this fragment should pass through the magnet as the resolving power was set to three decimal places. The major problem in the analysis of 1 or 2 ppb quantities at this time is quantification and reproducilibity. One part per billion is detectable based on the 2 part per billion spiked sample which had a 20 to 1 signal to noise ratio(figure 8). The ratio variance for samples and standard injections was only 10%. This means that the ratio of ions can be used to confirm and quantitate the toxin. With such a small sample size (2 gram blood) and low amounts injected (400 picograms) reproducibility suffered much. Problems may be due as well to sample injection (use of the syringe as well since the matrix was rather viscous. An internal deuterated standard or homolog would be necessary for quantifying these amounts of toxin.

On column chromatography had some benefits and draw backs in the analysis. It allowed for overall greatest sample through put into the mass spectrometer but peak broadening, column coating degradation, and increased tailing may severely limit its performance on particularly dirty samples. The SPLITLESS method of sample introduction (figures 8 through 12) gave excellent chromatography for analysis in this low concentration region. Interpretation of data: The data appeared to be as easy to interpret as that of normal selected ion analysis. The ratio consistency as mentioned above was quite satisfactory. The purpose in using this method of analysis (MRM) was to demonstrate the lack of interferences and sample limits of detection. Base lines such as those shown in these analyses are quite uncommon for mixed matrix analyses. We were quite pleased to eliminate other chemical noise from the base line. This procedure should also be compared to other such procedures as selected ion recording.

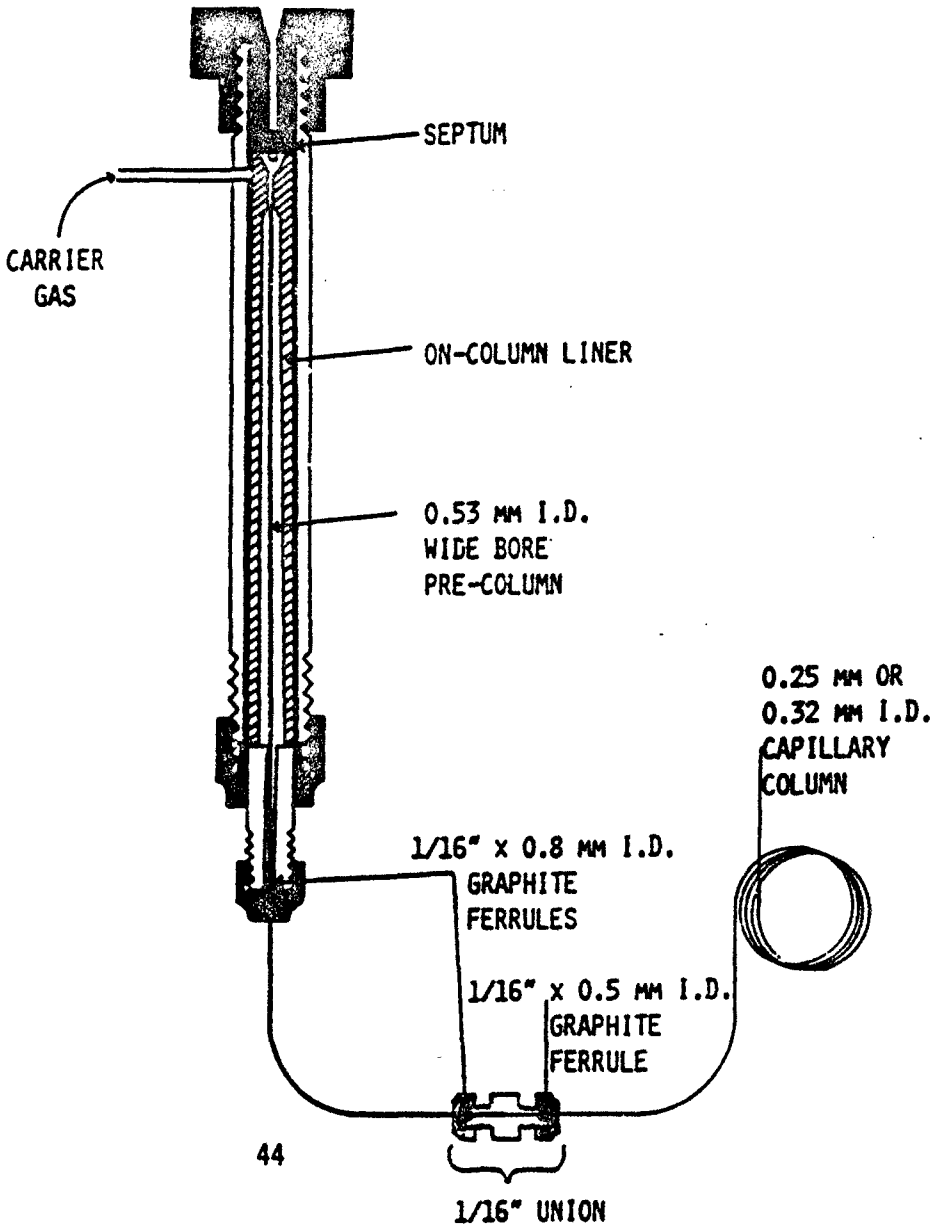
The splitless method of injection is demonstrated in figure 9 which shows the resolution of 2ppb of T-2-TFA in a biological matrix of the equivalent of 2ml of blood. The daughter monitored was m/z+ 180 which showed a minimum of noise (S/N = 17:1) . The daughters 138 and 121 are shown in figures 10 and 12 respectively. Their signal to noise ratios (12:1 and 14:1) are acceptable but inferior to that of 180. Our recommendation is that all three daughters be monitored for the purposes of calculating ratios but that 180 be used for quantitation.

Figure 12 shows the resolution of T-2-TFA in MRM using the

splitless method of injection. Injection of one nanogram under ideal conditions shows excellent resolution and peak width superior to that found in the On-Colum injection shown in figure 5.

We conclude from these studies that the SPLITLESS method of sample introduction into the GC/MS is the method of choice. It gives superior resolution when compared to ON-COLUMN injection. Mass fragment 478 should be used as the parent ion for analysis and the daughter 180 gives the best quantitative results. Fragment 478 was chosen after careful strudy of the daughters of 460, 461, 401 and 418. All of the above was done in the electron impact mode of analysis. The latter is traditionally less sensitive than positive chemical ionization (PCI) in methane which is our next effort in this study. We expect to increase our sensitivity by a factor of ten due to the large fragment ions encountered in PCI.

FIGURE 1. On-Column injection device used in the injection of the T-2 amended blood matrix through the Hewlett Packard gas chromatograph. This device consists of a 0.53 mm I.D. precolumn (about 15 cm in length) coupled to 0.25 mm I.D. 10 meter DB-5 analytical column. This method allows direct injection with a regular metal injection syringe rather than a flexible on-column injection syringe. The device is patterned on that of Hartman et al. of Rutgers University who has developed the procedure followed.



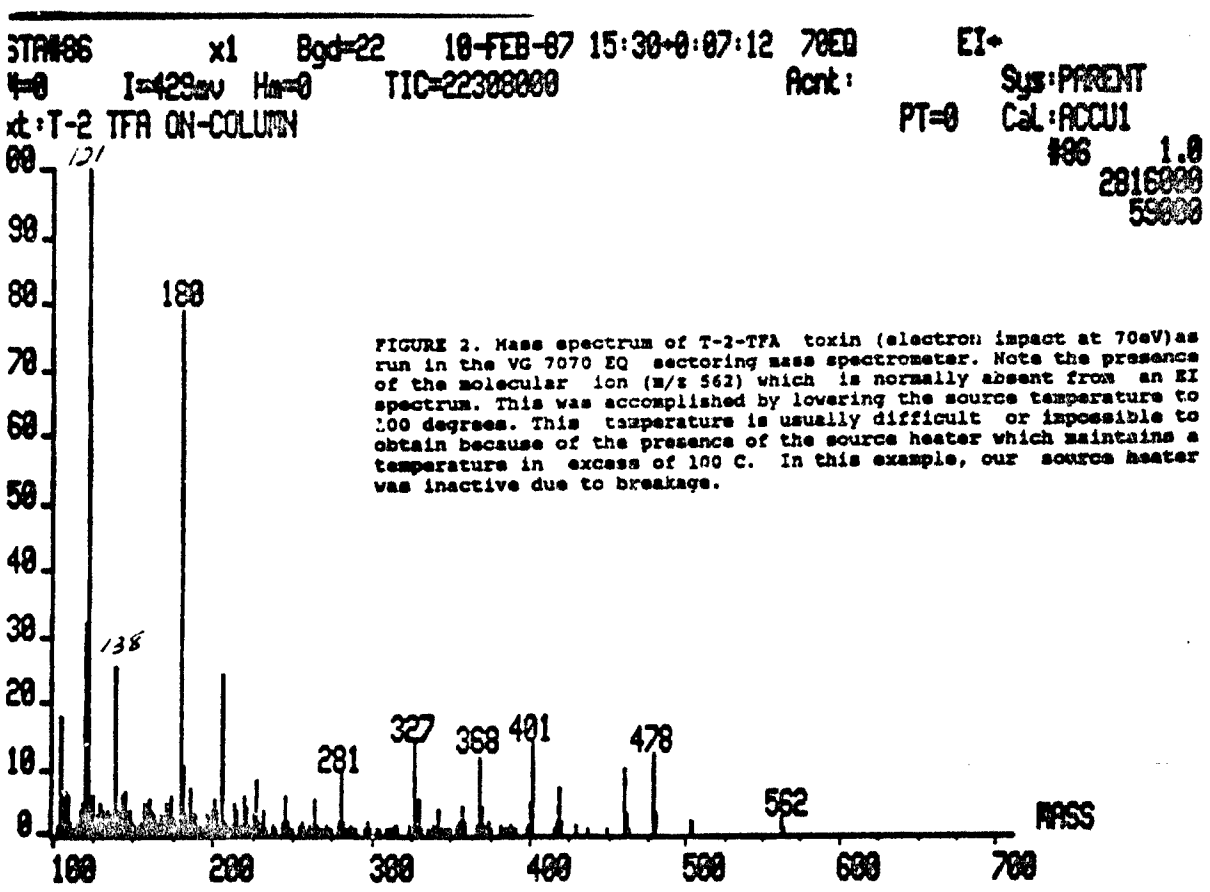
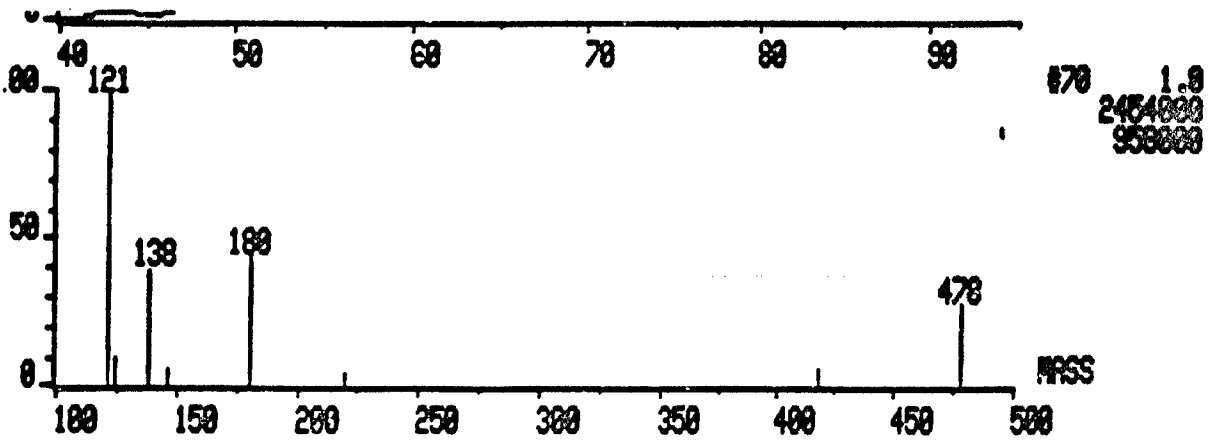


FIGURE 2. Mass spectrum of T-2-TFA toxin (electron impact at 70eV) as run in the VG 7070 EQ sectoring mass spectrometer. Note the presence of the molecular ion (m/z 562) which is normally absent from an EI spectrum. This was accomplished by lowering the source temperature to 100 degrees. This temperature is usually difficult or impossible to obtain because of the presence of the source heater which maintains a temperature in excess of 100 C. In this example, our source heater was inactive due to breakage.

FIGURE 3. Daughters ions obtained from fragmentation of ion 478 from the TFA derivative of T-2 toxin. Ions 180, 138 and 121 were chosen for subsequent MRM analysis. The fragment 478 was found to be superior (gave less background noise) to that of 401 in multiple reaction monitoring as determined in previous analyses.



```
* SYSTEM:MRM        Parameters for Selective Ion Recording [G# 1 MRM] (Sector)

DAT  Data filename                C:TESTB     IAV  Maximum volts                6000
REF  Reference filename             PFK       IMR  Maximum mass at IAV          2256
INS  Instrument                   1:70EQ      GTM  Time 0:06:00 0:20:00 Mode EI+B Gas
ACN  Customer account                         CHN  M(amu) S(ms) D(ms)     M(amu) S(ms) D(ms)
ACV  Accelerating volts           6000         478   180    100    10
RES  Instrument resolution        1000         478   138    100    10
SMP  #Samples                        1         478   121    100    10
INJ  #Injections                     1
GRP  #Groups                         1
CLS  #Calibration scans              0
CST  Cal. scan time (s)              5
CTL  Cal. tolerance (ppm)          200
CEX  Cal. examination                Y
PEX  Peak examination                Y
LMS  Lock span (peak widths)       2.0
LST  Lock step (peak widths)      0.02
FLO  Fast lock on                    N

TXT  Sample#  1: 500 PICO T-2 TFA DEV. MRM ON-COLUMN

'H=hardcopy RETURN=next ESC=prev CTRL/A=abort
'G=go 'Q=quit 'C=create 'DEL=delete 'O=overwrite 'Z=zero <group,sample>
```

FIGURE 4. Multiple Reaction Monitoring Parameter Page For T-2 toxin TFA Derivative. The exact parent ion mass of 478.145 was selected. The daughters monitored were 180,138, and 121. .PA

```
ESTB      11-FEB-87 21:40 70EQ      Acnt:                Sus:MRM
R 1  A: 478.1450  B: 478.1450  C: 478.1450
ext: T-2 TFA MRM ON-COLUMN
```

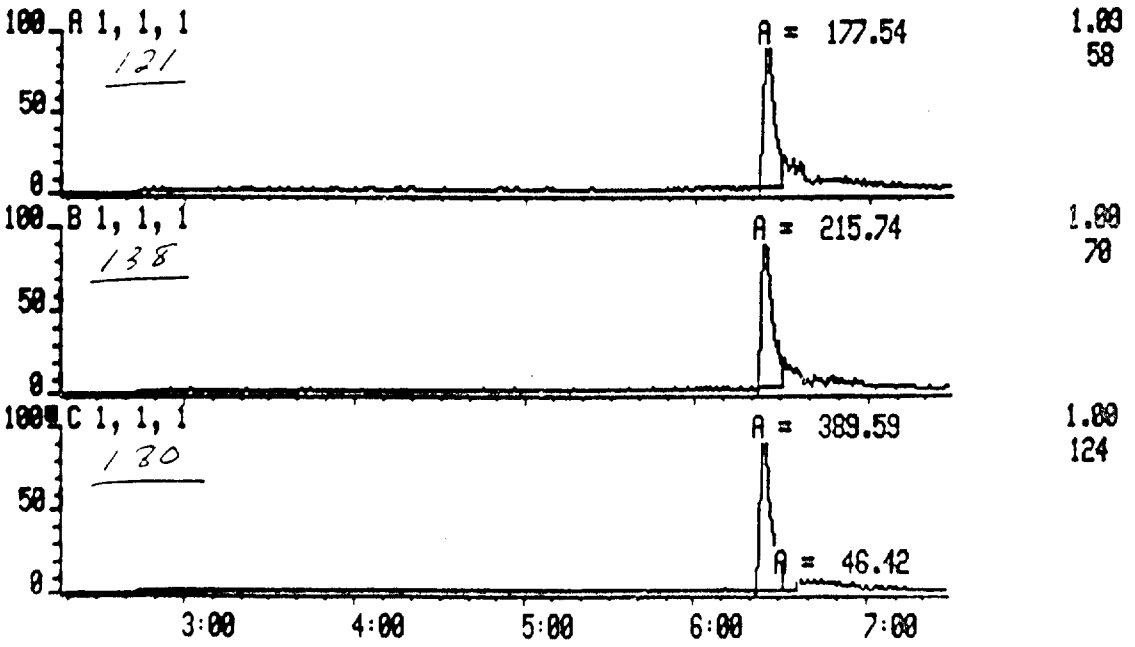


FIGURE 5. Ratios and retention time of T-2 TFA pure standard. Retention time 6.28 minutes. Ions 121/180 0.46, ions 138/180 0.55 taken from parent ion 478 going to ions 121, 138, and 180. Oncolumn injection. 10 meter 0.25 capillary column. GC oven temperature program 80/300 at 30 deg. per sec.

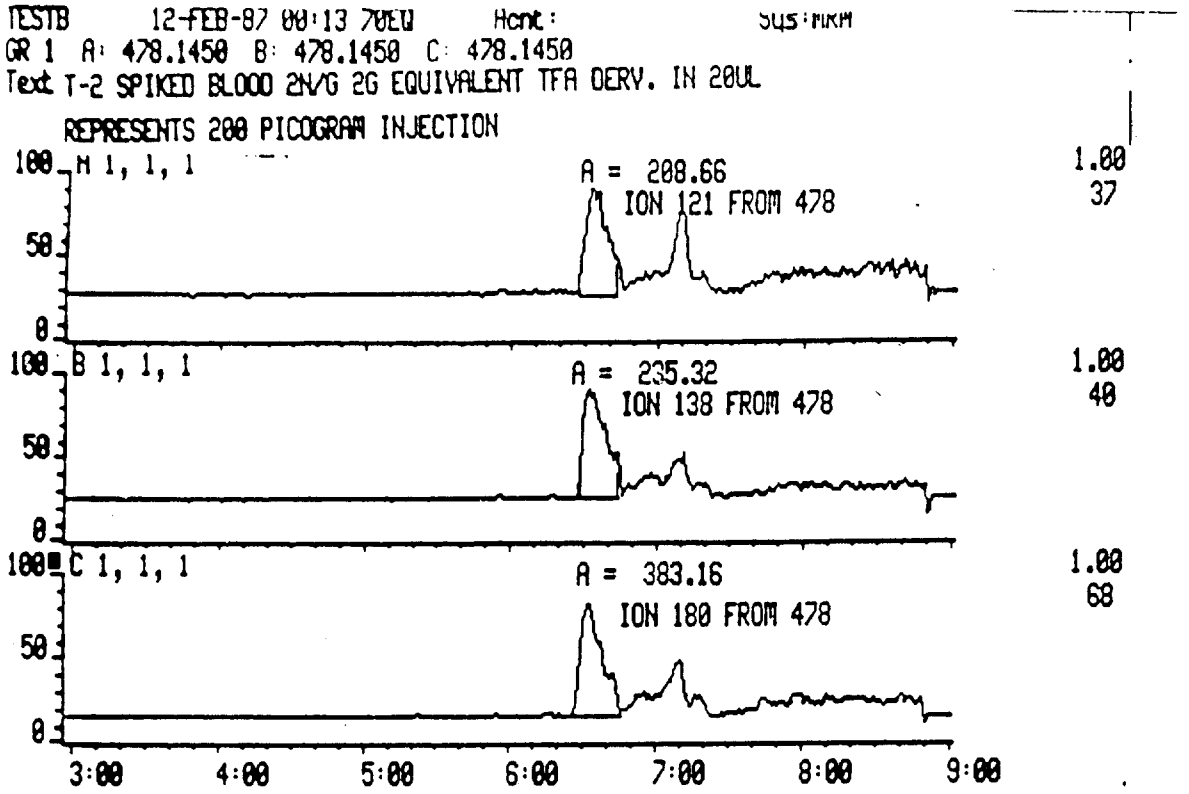


FIGURE 6. Multiple reaction monitoring (MRM) of a 2ml blood sample amended with 2ppb of T-2 toxin. The sample was introduced into the GC/MS via ON-COLUMN INJECTION. This method of injection will be contrasted with splitless injection in a later section.

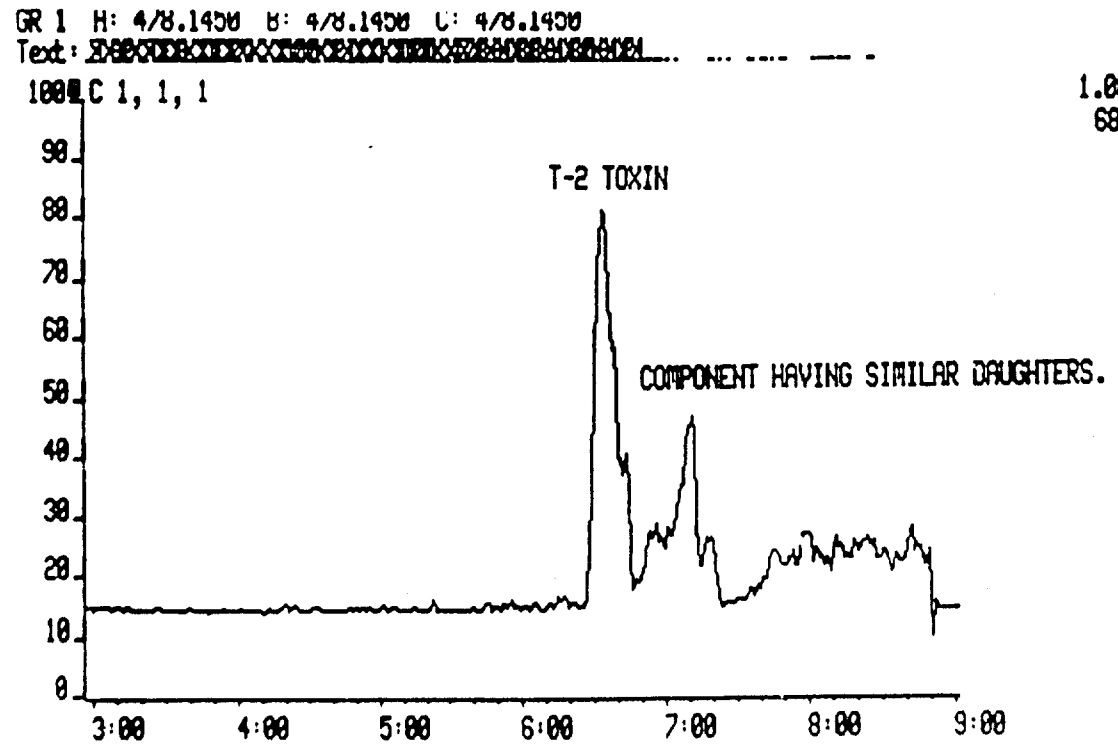


FIGURE 7. Component found in donor blood having similar properties to that of T-2 toxin except that its retention time is different(Rt=6.35 for T-2 vs 7.) for the unknown). The daughters of the unknown have different ratios from that of T-2 (T-2= 121/180=0.46 and 138/180=0.55)(ratios of unknown= 121/180=1.36 and 138/180=0.76). The unknown component has a significant m/z=478 similar to T-2-TFA so that when the magnet is parked on 478, both the T-2 and the unknown component are passed through.

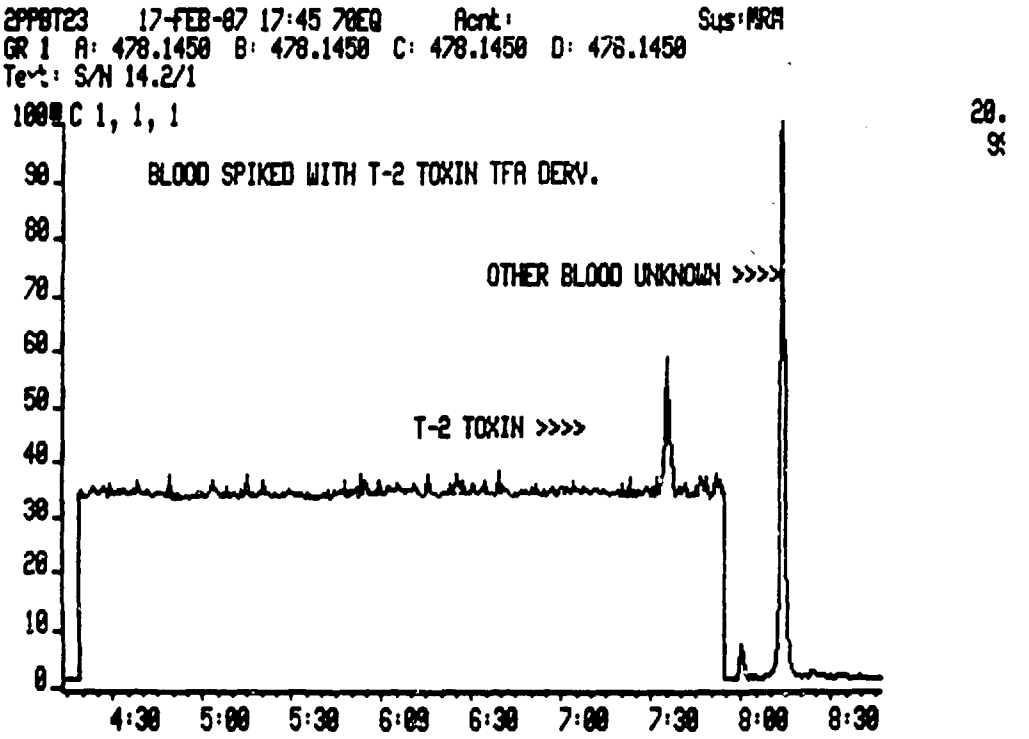


FIGURE 8. Resolution and quantitation (2ppb in blood matrix) of T-2 TFA toxin by MRM using the SPLITLESS method of compound introduction. Note the 10 second peak width at the base which is a great improvement over the ON-COLUMN injection. The unknown metabolite in the blood sample can be seen at Rt 8.3; its resolution has also been increased. Compare with resolution as shown in figures 6 and 7.

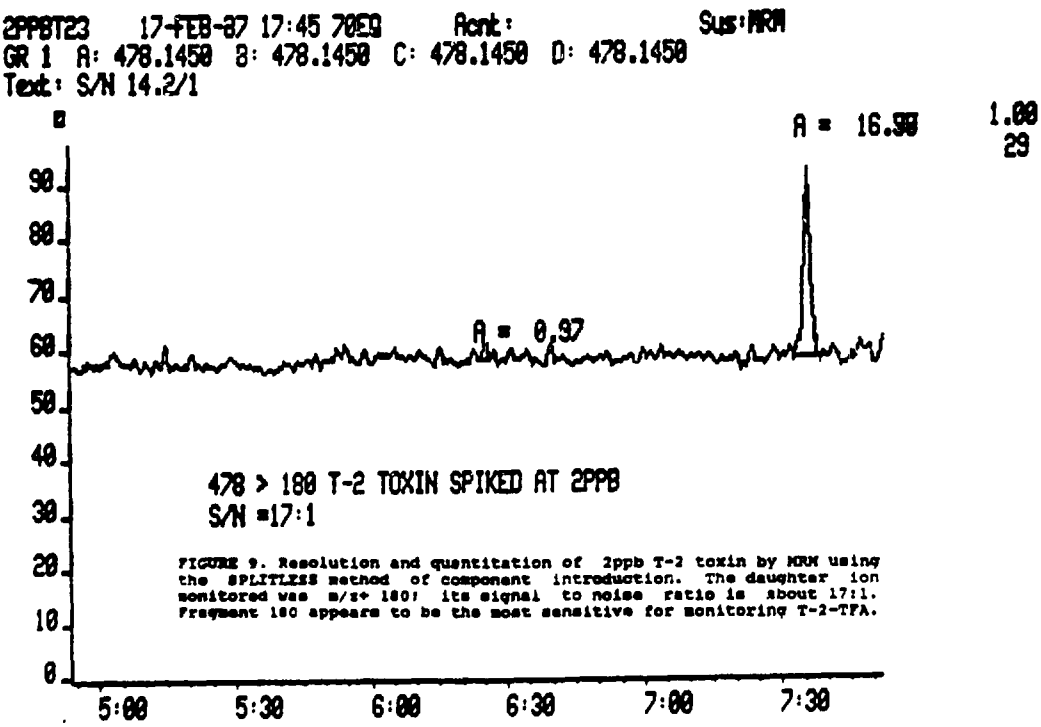


FIGURE 9. Resolution and quantitation of 2ppb T-2 toxin by MRM using the SPLITLESS method of componant introduction. The daughter ion monitored was m/z+ 180; its signal to noise ratio is about 17:1. Fragment 180 appears to be the most sensitive for monitoring T-2-TFA.

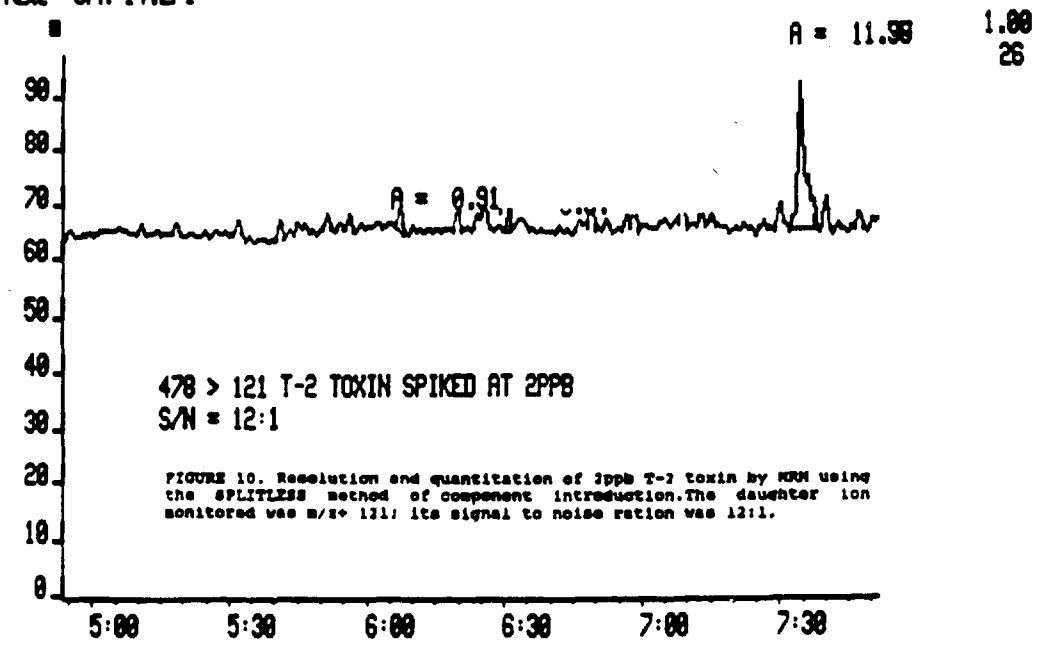


FIGURE 10. Resolution and quantitation of 2ppb T-2 toxin by MRM using the SPLITLESS method of component introduction.The daughter ion monitored was m/z+ 121; its signal to noise ration was 12:1.

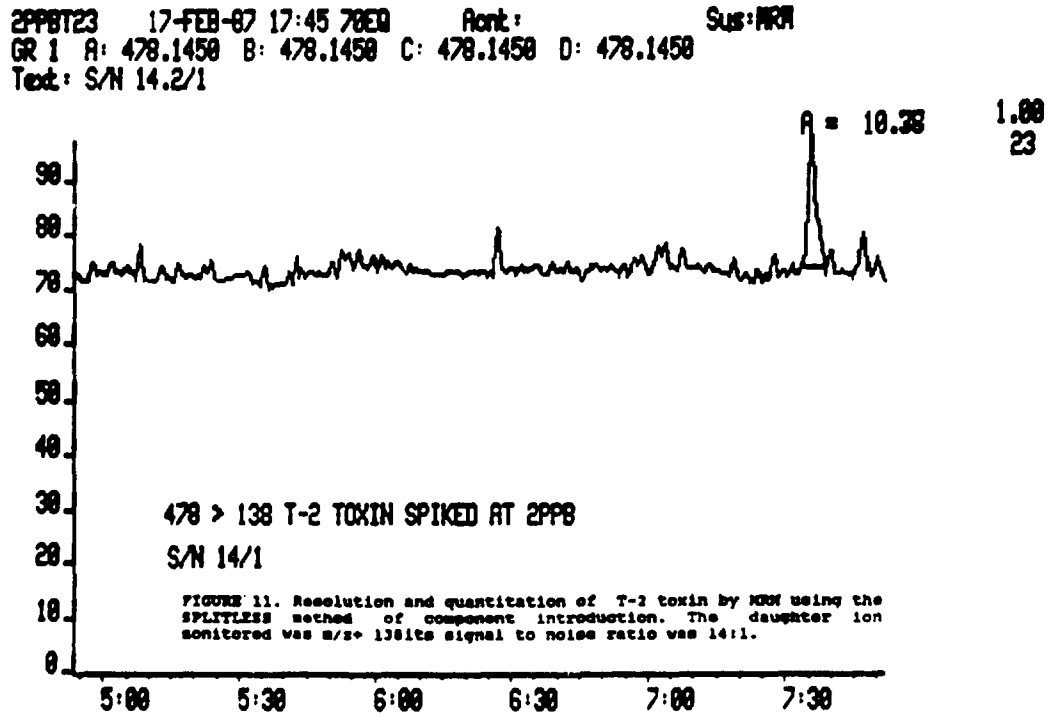


FIGURE 11. Resolution and quantitation of T-2 toxin by MRM using the SPLITLESS method of component introduction. The daughter ion monitored was m/z+ 138its signal to noise ratio was 14:1.

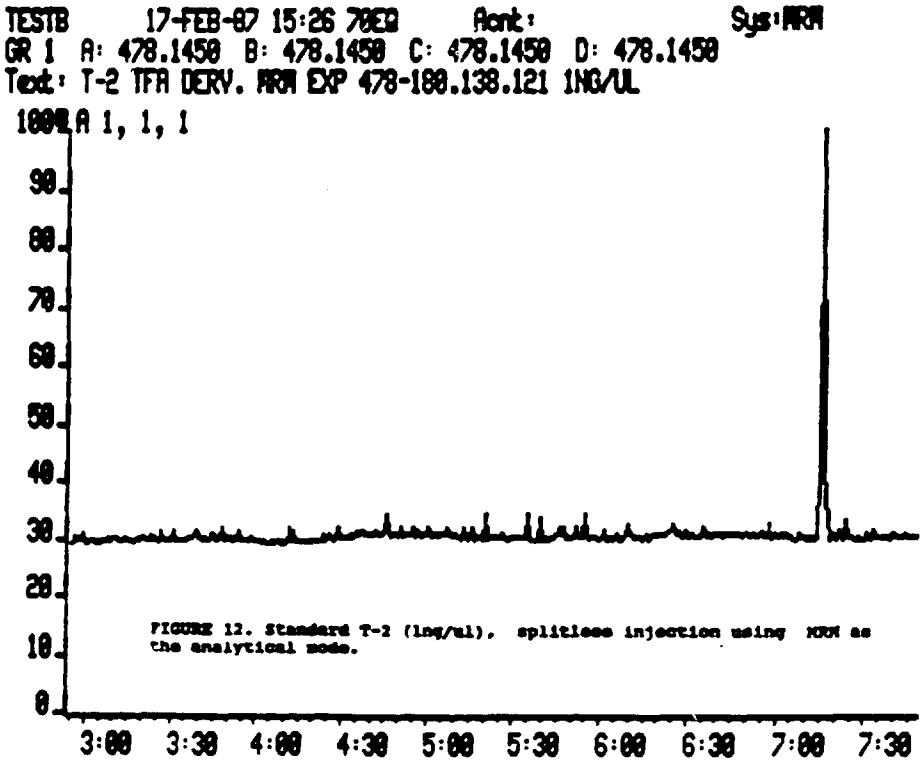


FIGURE 12. Standard T-2 (1ng/ul), splitless injection using MRM as the analytical mode.

TOXICITY AND TOXINS PRODUCED BY ISOLATES OF FUSARIUM SPECIES OBTAINED FROM NORWAY AND NEW ZEALAND

Table 1 shows the complete record of the toxicity of the isolates of Fusarium obtained from Norway in 1984. The collections were made from the Arctic as well as the central and southern regions of that country. The species most prevalent were Fusarium acuminatum, F. avenaceum, F. oxysporum and F.sambucinum. From these groups, F. oxysporum and F. sambucinum emerged as the most toxic although toxicity was also found among fewer isolates of F.acuminatum and F.avenaceum.

All of the toxic isolates shown in table 1 were saved and examined for the kinds and quantities of known toxins they produce in order to account for the toxic lesions. The toxins that were analyzed for are: trichothecenes; HM-8, a cytotoxic unknown Fusarium metabolite presently being characterized in our laboratory; moniliformin; Fusarin-C; and H-1 the toxic hemorrhagic agent presently being characterized in our laboratory.

Table 2 shows the various known toxins produced by the various isolates of Fusarium in an effort to account for their toxicity. Within the F. acuminatum group, the predominant toxins found were HM-8 and moniliformin. F. avenaceum produced HM-8, moniliformin and infrequently Fusarin-C; only one isolate produced the hemorrhagic agent know as H-1. Although a considerable portion of the isolates of F. oxysporum yielded HM-8 and moniliformin, the majority of the toxicity could be accounted for by H-1. It is curious to note that none of the isolates that produced H-1 produced HM-8 as well and vice versa. Within the taxonomic group known as F. sambucinum, the largest percentage produced H-1. Only one isolate produced HM-8 and H-1 together.

Table 3 shows a compilation of the various toxins that were found in the toxic groups of Fusarium. In these analyses, TDP-1 (fusarochromanone) was also looked for. TDP-1 is the Fusarium metabolite that causes bone disorders. None of the isolates investigated produced TDP-1 whereas the majority of the F. sambucinum isolates produced H-1. The F. oxysporum group competed well with F. sambucinum for the production of H-1; however, the members of this group also produced moniliformin but not simultaneously with H-1. The significance of this observation is not apparent at present. The production of moniliformin was well represented among F. acuminatum and F. avenaceum.

An effort was made to determine whether the H-1 producing isolates could be found in regions outside of Norway. A collection was made in Tara Hills, a mountainous region in the South Island of New Zealand. Six isolates , all of them belonging

to the Fusarium sambucinum group, produced this toxin (Table 4). The Tara Hills are characterized as high grassy hills some of which receive a lot of shade; the climate is cold in the winter. The sheep grazing in this area become unthrifty and often called "poor doers" and this was particularly associated with the shady region of the Tara Hills complex. It is possible that Fusarium colonizing the grass may influence the health or these animals by the production of toxins such as H-1.

The isolates examined in Table 4 were selected because of their toxicity to rats, our bioassay test animal. Toxicity was accounted for by those isolates (6) that produced H-1, four isolates that produced moniliformin, and the six isolates that produced fusarenone-x. The toxins found ranged from deoxynivalenol to 3-acetyldeoxynivalenol, HM-8, zearalenone,moniliformin and H-1. Isolate 6F4 is being studied to identify the toxic component accounting for death in the test animals. This isolate produced DON, 3-acetyl-DON and zearalenone; these do not account for the toxicity.

Table 1. Toxicity of *Fusarium* isolates in rats fed a 1:1 mixture of *Fusarium* culture (rice) and complete rat diet.

Fusarium species	# isolates	# isolates causing					Food consumption (g)[c]
		Death	Hemorrhage[a]	Wt. loss (-)	No change (0)	Wt. gain (+)	
Control 1 (n=30)	-	0	0	0	0	30	43.13 ± 3.01
Control 2 (n=30)	-	0	0	0	0	21	41.33 ± 1.10
F. acuminatum	25	4	0	23	0	2	23.22 ± 3.13
F. avenaceum	28	7	3	20	1	7	20.51 ± 3.08
F. culmorum[b]	1	0	0	1	0	0	17.30 ± 3.64
F. oxysporum	31	25	19	28	0	3	10.55 ± 2.74
F. sambucinum	40	37	33	40	0	0	7.88 ± 1.46
Total # isolates	125	73	55	112	1	12	

[a] Includes hemorrhage of stomach, intestine, thymus, as well as hematuria.

[b] This isolate caused uterine enlargement.

[c] Each value is the mean for the total number of rats used for each species ± standard deviation.

Table 2. Production of mycotoxin from Fusarium isolates grown on rice medium.

Fusarium species and code number	Source	Area of origin	Mycotoxins*
Control 1	Complete rat diet.	-	0
Control 2	Autoclaved rice-complete diet(1:1)	-	0
F. acuminatum:			
01A	Grassfield	Leksele	+/-0
01FC	Cultivated grass	Leksele	+/-0,Mo
02EA	Cultivated grass	Ovre Grunsele	+/-0,Mo
02EB	Cultivated grass	Ovre Grunsele	+/-0,Mo
02EC	Cultivated grass	Ovre Grunsele	+/-0,Mo
02JA	Cultivated grass	Ovre Grunsele	Mo
02JB	Cultivated grass	Ovre Grunsele	Mo
02JC	Cultivated grass	Ovre Grunsele	Mo
02SA	Oats	Fossestrand	+/-0,Mo
02SB	Oats	Fossestrand	+/-0,Mo
02SC	Oats	Fossestrand	+/-0,Mo
02UA	Oats	Fossestrand	Mo
02UB	Oats	Fossestrand	Mo
02UC	Oats	Fossestrand	Mo
02UC(2)	Oats	Fossestrand	0
03BA	Oats	Osterbaek	+/-0,Mo
03BB	Oats	Osterbaek	+/-0,Mo
03BC	Oats	Osterbaek	0

Table 2. (Continued)

Fusarium species and code number	Source	Area of origin	Mycotoxins
F. acuminatum:			
03IA	Oats	Osterbaek	Mo
03IB	Oats	Osterbaek	0
03N4	Barley	Alta	Mo
04ZA	Timothy	Tromso	Mo
04ZB	Timothy	Tromso	+/-0,Mo
077	Rhodin	Ullsberg	+/-0,Mo
086	Rhodin	Opplond County	+/-0
F. avenaceum:			
02BA	Oats	Fossestrand	Mo
02BB	Oats	Fossestrand	F-C
02DA	Oats	Osterbaek	+/-0,Mo
02DB	Oats	Osterbaek	+/-0,Mo
02DC	Oats	Osterbaek	+/-0,Mo
03JC	Timothy	Alta	0
03MB	Barley	Alta	Mo
040A	Barley	Alta	+/-0,Mo
040B	Barley	Alta	Mo
040C	Barley	Alta	Mo
041A	Barley	Alta	Mo
041B	Barley	Alta	0
041C	Barley	Alta	Mo

Table 2. (Continued)

Fusarium species and code number	Source	Area of origin	Mycotoxins
F. [illegible]			
06JA	Barley	[illegible]	+/-0,Mo
06JB	Barley	[illegible]	+/-0,Mo
06J6	Barley	[illegible]	+/-0,Mo
06BA	Dactylis glomerata	[illegible]	+/-0,Mo
06BB	Dactylis glomerata	[illegible]	Mo
060	Barley straw	[illegible]	+/-0
07BA	Overwintered barley	Evitehamar	+/-0,Mo
07BB	Overwintered barley	Evitehamar	Mo
07BC	Overwintered barley	Evitehamar	Mo
07DA	Overwintered barley	Telemark County	+/-0,Mo
079	Rhodin	Skog	Mo
08BA	Natural grass	Sunndal	+/-0
08BB	Natural grass	Sunndal	+/-0
08BC	Natural grass	Sunndal	+/-0
093A	Timothy	Opplond County	+/-1
F. [illegible]			
096EL(2)**	Barley	Tromso	F-2
F. [illegible]			
010	Grassfield	Leksele	0-1
02A	Grass area	Leksele	+/-0,Mo
011A	Cultivated grass area	Leksele	+/-0,Mo

Table 2. (Continued)

Fusarium species and code number	Source	Area of origin	Mycotoxins
F. [illegible]			
011B	Cultivated grass area	Leksele	+/-0,Mo
012A	Cultivated grass area	Leksele	+/-0,Mo
012B	Cultivated grass area	Leksele	+/-0,Mo
012C	Cultivated grass area	Leksele	+/-0,Mo
013B	Cultivated grass area	Leksele	+/-0,Mo
017A	Grass area	Leksele	0-1
017B	Grass area	Leksele	0-1
02GA	Cultivated grass	Ovre Grunsele	+/-0
02GB	Cultivated grass	Ovre Grunsele	+/-0,Mo
02GC	Cultivated grass	Ovre Grunsele	+/-0,Mo
02IA	Cultivated grass	Ovre Grunsele	+/-0,Mo
02IB	Cultivated grass	Ovre Grunsele	Mo
02IC	Cultivated grass	Ovre Grunsele	+/-0,Mo
033A	Barley	Alta	0-1
033B	Barley	Alta	0-1
038A	Barley	Alta	0-1
039A	Timothy	Alta	0-1
039B	Timothy	Alta	0-1
039C	Timothy	Alta	0-1
040A	Barley	Tromso	0-1
040C	Barley	Tromso	0

Table 2. (Continued)

Fusarium species and code number	Source	Area of origin	Mycotoxins
F. oxysporum:			
[illegible]	Overwintered barley	[illegible]	0
[illegible]	Overwintered barley	[illegible]	Mo
[illegible]	Overwintered barley	[illegible]	Mo
[illegible]	Overwintered barley	[illegible]	UN-0,Mo
[illegible]	Overwintered barley	[illegible]	UN-0,Mo
[illegible]	Overwintered barley	[illegible]	N-1
[illegible]	Everlasting	Upland County	N-1
F. sambucinum:			
[illegible]	Barley	Alta	N-1
[illegible]	Barley	Alta	N-1
[illegible]	Barley	Alta	N-1
[illegible]	Barley	Alta	N-1
[illegible]	Timothy	Alta	N-1
[illegible]	Timothy	Alta	0
[illegible]	Timothy	Alta	N-1
[illegible]	Timothy	Alta	0
[illegible]	Barley	Trenton	N-1
[illegible]	Barley	Trenton	N-1
[illegible]	Barley	Trenton	0
[illegible]	Barley	Trenton	0
[illegible]	Timothy	[illegible]	N-1

Table 2. (Continued)

Fusarium species and code number	Source	Area of origin	Mycotoxins
F. sambucinum:			
[illegible]	Onion field	[illegible]	N-1
[illegible]	Onion field	[illegible]	Unknown
[illegible]	Barley field	[illegible]	N-1
[illegible]	Barley field	[illegible]	N-1
[illegible]	Barley field	[illegible]	N-1
[illegible]	Barley field	[illegible]	N-1
[illegible]	Overwintered barley	[illegible]	UN-0
[illegible]	Meadow	Upland County	N-1
[illegible]	Meadow	Upland County	N-1
[illegible]	Meadow	Upland County	N-1
[illegible]	Meadow	Upland County	N-1
[illegible]	Natural grass	Upland County	N-1
[illegible]	Natural grass	Upland County	N-1
[illegible]	Natural grass	Upland County	N-1
[illegible]	Overwintered barley	Teleware County	UN-0,N-1
[illegible]	Natural grass	[illegible]	N-1
[illegible]	[illegible] grass	Upland County	N-1
[illegible]	[illegible] grass	Upland County	N-1
[illegible]	Everlasting	Upland County	N-1
[illegible]	[illegible]	Upland County	N-1
[illegible]	[illegible]	Upland County	N-1

Table 2. (Continued)

Fusarium species and code number	Source	Area of origin	Mycotoxins
F. sambucinum: (Continued)			
[illegible]	[illegible]	Upland County	N-1
[illegible]	Timothy	Upland County	N-1
[illegible]	Mountain heather	Upland County	N-1
[illegible]	Mountain heather	Upland County	N-1
[illegible]	[illegible]	Upland County	N-1
[illegible]	[illegible]	Upland County	N-1

* 0 = No mycotoxin detected; UN-0 = unknown non-trichothecene mycotoxin produced by *Fusarium* spp. (m.w. 226, m.p. 181-182°C); Mo = Moniliformin; F-c = Fusarin C; N-1 = unknown non-trichothecene mycotoxin produced by *Fusarium* spp. (m.w. [illegible], m.p. [illegible]°C); and F-2 = zearalenone.

Table 3. Mycotoxins detected in extracts of fungal isolates used in these studies.

Fusarium species	Number isolates	Number of isolates producing mycotoxins						
		Trichothecenes	F-2	Fusarin c	TDP-1	HN-8*	H-1**	Mo
F. acuminatum	25	0	0	0	0	13	0	20
F. avenaceum	28	0	0	1	0	14	1	20
F. culmorum	01	0	1	0	0	0	0	0
F. oxysporum	31	0	0	0	0	14	12	17
F. sambucinum	40	0	0	0	0	2	34	1
Total no. of isolates	125	0	1	1	0	43	47	58

* Metabolite of unknown structure produced by *Fusarium* spp. (M.W. 226, M.P. 181-182°C).

** Metabolite of unknown structure produced by *Fusarium* spp. (M.W. 428, M.P. 222-223°C).

Code #	Fusarium Species	Area of origin	Death of rat within the feeding test	DON	3 ac-DON	Toxin HH-8	F-X	F-2	Monilif.	H-1
2 F_{63} F_9	F. avenaceum	Pasture leaves	1	x?			x		x	
1 F 2	F. culmorum	South Auckland	1	x			x	x		
9 FA_{10}	F. culmorum	South Auckland	3	x			x	x		
6 F_4	F. oxysporum	South Auckland	3	x	x			x		
8 FA_{10}	F. oxysporum	South Auckland	1	x		x*		x*	x	
9 FB_4	F. oxysporum	South Auckland	-					x	x	
9 FB_6	F. sambucinum	South Auckland	1	x			x	x		
3 A	F. sambucinum	Tara-high wet stocking	1							x
4 A	F. sambucinum	Tara-shade	1							x
5 A	F. sambucinum	Tara-shade	3	x?						x
6 A	F. sambucinum	Tara-random	1							x**
5 B	F. sambucinum	Tara-shade	3	x?						x
1 D	F. sambucinum	Tara-shade	1							x
HS 1 A	F. sambucinum	Pukete Rd	1				x	x**	x	
HS 4 A	F. sambucinum	Pukete Rd	-			x?	x	x**		

TABLE 4. Mycotoxins found in fifteen toxic isolates of Fusarium collected in New Zealand.

The extraction of the culture material was done with MeOH /H_2O, ratio 55:45.
The toxins were only confirmed by DC (TLC plates - kieselgel 60, Merck 20 x 20 cm layer thickness 0.25 mm).

Developing systems were - chloroform/Methanol, ratio 9:1 and 4:1
- chlorofrom/Aceton, ratio 3:1

Spraying reagent were - p - anisaldehyd
- 20% H_2 So_4
- 4 - (4 - Nitrobenzyl) purdine together with tetraethylene - pentamine.
- 20% AL Cl_3 · H_2O
- 0.32% (w/r) aqueous 2,4 dinitrophenylhydrazine

Standards used for comparison: MAS, DAS, T-2, acethyl - T-2, HT-2, T-2 tetraol, DON, 3 acetyl-DON, 15 acetyl - DON, F-X, Nivalenol, HM-8, acetyl - HM-8, F-2, Moniliformin, H-1, TDP -1

Code: x? & not sure

x* & much

x** & very much

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